A role for SOX9 and Wnt-related proteins in endometrial adenocarcinoma

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by

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ABSTRACT

A ROLE FOR SOX9 AND WNT-RELATED PROTEINS IN ENDOMETRIAL ADENOCARCINOMA

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Introduction: Endometrial carcinoma is already the most common gynaecological malignancy in the developed world, and by 2015 the incidence is set to be twice that of 2005. Despite this, therapeutic options available for advanced disease are very limited. The canonical Wnt/β-catenin pathway is known to have a key role in homeostasis of normal endometrial cellular proliferation and differentiation, and is also implicated in endometrial proliferative conditions such as carcinogenesis. SOX9 is a postulated Wnt-regulator and may play a key role in establishment and maintenance of characteristic phenotypes of glandular cells during the menstrual cycle. The interaction of Wnt/β-catenin pathway and SOX9 in the endometrium is not known, neither are the effects of ovarian steroids on these pathways and proteins fully elucidated. The expression of a panel of Wnt-related proteins in endometrial cancer tissue samples was compared to normal pre- and post-menopausal endometrium. Further in vitro work investigated the effect of ovarian steroid hormones on SOX9 and steroid receptor expression in a well characterised, well differentiated endometrial carcinoma cell line Ishikawa.

Methods: 51 primary endometrial samples (31 endometrial adenocarcinomas (EC), 10 normal proliferative phase (PP) and 10 normal post-menopausal (PM)) were assessed for the expression of SOX9, Ki67, β-catenin, SOX2, SFRS2, and NAP1L1 using immunohistochemistry. The Ishikawa cell line was maintained in phenol-free charcoal stripped media for 72 hours, and then treated with ovarian steroids: *estradiol* (E_2), medroxyprogesterone acetate (MPA), E_2 + MPA, basic fibroblast growth factor (FGF), and 5-alpha-dihydrotestosterone (DHT) for 72 hours. RT-PCR was performed for oestrogen receptor α (ER), progesterone receptor (PR), and SOX9. Statistical analysis used the Mann-Whitney and Spearman Rank tests.

Results: Nuclear β-catenin was correlated with the Wnt-related proteins SOX2 ($p=0.025$), NAP1L1 ($p=0.046$) and SFRS2 ($p=0.032$). However, none of these proteins demonstrated significantly different expression across the groups. The expression of SOX9 was significantly lower in the proliferative tissues (PP and EC) than the atrophic PM ($p<0.0001$). Correspondingly Ki67 was significantly lower in the PM samples than the PP and EC ($p<0.0001$). SOX9 and Ki67 also demonstrated an inverse relationship ($p=0.032$). ER expression was not altered by any of the ovarian steroid hormones. PR was up-regulated by E_2 (1.8 fold, $p=0.036$), E_2+MPA (1.5 fold, $p=0.071$) and DHT (1.9 fold, $p=0.036$) from baseline. SOX9 was down-regulated 1.5-fold by both MPA ($p=0.036$), FGF ($p=0.036$) and DHT (not statistically significant).

Discussion: These results provide further evidence for the pivotal role played by Wnt in the normal and malignant endometrium. This also forms the first report identifying the Wnt-related proteins NAP1L1 and SFRS2 in the malignant endometrium. SOX9 is differentially expressed between proliferative and atrophic endometria, and can be down-regulated by MPA, FGF and DHT. This therefore provides preliminary data on a role for androgens in the endometrium, since DHT was demonstrated to alter expression of both PR and SOX9. Androgens are the dominant hormone in the post-menopausal endometrium, from which endometrial cancer almost exclusively arises; further work expanding our understanding of androgens is therefore vital. Novel treatment strategies manipulating the Wnt pathway using androgens can be envisaged to treat this common cancer in the future.

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Abbreviations

APC: Adenomatous polyposis coli

APES: Aminopropyl triethoxy silane

BMI: Body mass index

BP: Base pairs

BSA: Bovine serum albumin

CIN: Cervical intraepithelial neoplasia

cDNA: Complementary deoxyribonucleic acid

DHT: 5 alpha-dihydrotestosterone

E_2: β-estradiol

EDTA: Ethylenediaminetetraacetic acid

EMT: Epithelial to mesenchymal transition

ER: Oestrogen receptor

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FIGO: International Federation of Gynaecology and Obstetrics

GCP: Good clinical practice

HMG: High-mobility group

HRP: Horseradish Peroxidase labelled polymer

IHC: Immunohistochemistry

IgG: Immunoglobulin G

KW: Kruskal Walllis statistical test

LREC: Local research ethics committee

KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

MPA: Medroxyprogesterone acetate

MW: Mann Whitney statistical test

NAP1L1: Nucleosome assembly protein 1-like 1

NBF: Neutral buffered formalin

PCNA: Proliferating cell nuclear antigen

PCR: Polymerase chain reaction

PM: Post-menopausal

PP: Proliferative phase

PR : Progesterone receptor

PTEN: Phosphatase and tensin homologue

RLUH: Royal Liverpool University Hospital

RNA: Ribonucleic acid

SC35: Splicing factor 35

SD: Standard deviation

SR: Serine/arginine-rich

SFRS2: Serine/arginine-rich splicing factor 2

SOX: Sex-determining region Y box

TAE: Tris-Acetate EDTA

TBS: Tris buffered saline

TP53: Tumour protein 53

Chapter 1. Introduction

The endometrium is arguably the most plastic of the adult human organs. Unfortunately, the mechanisms which lend it this remarkable ability to regenerate are also implicated in the endometrial proliferative conditions. Endometrial adenocarcinoma is the most common gynaecological malignancy, and whilst its incidence rises at a startling rate, survival rates are static, resulting in a rising death toll. In addition to regulating the fine balance between differentiation and proliferation in the normal cycling endometrium, the canonical Wnt/β-catenin pathway is increasingly thought to be a key factor in endometrial carcinogenesis. A thorough review of the currently available literature was conducted of the structure and function of the endometrium, endometrial adenocarcinoma and Wnt-related protein in the endometrium.

1.1. The human uterus and endometrium

1.1.1 Anatomy

The human uterus lies at the centre of the female reproductive tract; it is a highly muscular, hollow, pear shaped organ measuring approximately 8 x 5 x 3 cms[8] (Figure 1). Its primary function is the vital role of enabling the development of the embryo to fetus following migration of the ovum from the ovaries to the uterus via the fallopian tubes. It also serves as a conduit for migrating sperm[2]. The uterus comprises of a fundus, body and cervix. The fallopian tubes protrude superiorly from the uterus,

and inferiorly the cervix opens into the vaginal vault via the external os[9]. The uterus lies in the pelvic cavity, approximately in the midline, with close relations to both the urinary bladder and rectum[8]. Embryologically, the female reproductive tract is derived from mesoderm; the two Müllerian (paramesonephric) ducts differentiate into oviducts, uterus, cervix and upper portion of the vagina[3].

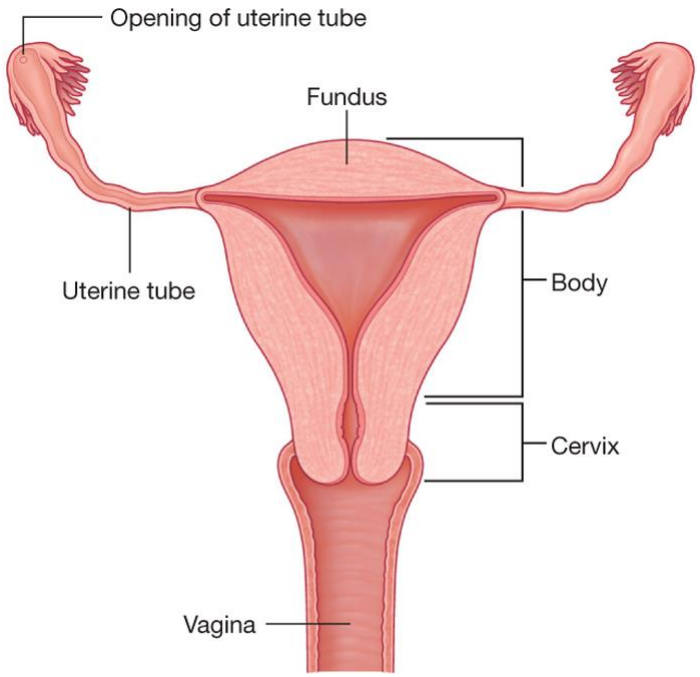


Figure 1: Anatomy of the female reproductive tract. Adapted from Drake *et al.*.[2]

The blood supply for the uterus, and therefore the endometrium is provided by the arcuate arteries which arise from the uterine and ovarian arteries[9]. The radial arteries arise from these in the myometrium. Crossing from the myometrium to the endometrium, they branch to form the basal and spiral arteries which are anastomosing and terminal arteries respectively. The functional layer is supplied by the branching spiral arteries which form a sub-epithelial plexus just below the surface, draining into venous sinuses[10].

The vasculature within the endometrium must undergo cycles of growth and regression due to the menstrual cycle, which makes it unique in the human body. During the proliferative phase, the growth of the functionalis is accompanied by similar growth in the vascular tree. By the end of this phase, the terminal branches have become coiled, and these spiral arteries ascend from the basal to the functional layer during the secretory phase[10].

Structurally the uterus is composed of two layers; the thick muscular fibro-elastic myometrium[8], and the inner endometrium which is arguably the most plastic organ within the human body. In preparation for implantation of a fertilised blastocyst, the endometrium must be fully re-organised monthly following menstruation. This occurs under the influence of cyclical variations in sex steroid hormones, the mechanism of which involves a wide variety of local autocrine and paracrine actions including enzymes, hormones and bioactive peptides[10]. The precise mechanisms of some of these actions are far from being fully defined.

1.1.2 Endometrium

The endometrium of the uterine corpus is composed of a lower basal layer (stratum basalis) adjacent to the myometrium, and the superficial functional layer (stratum functionalis)[3]. The functionalis regenerates monthly to accommodate the blastocyst should fertilisation occur, and subsequently provide the maternal portion of the placenta during pregnancy[3]. The basalis is the putative location of the endometrial

stem or progenitor cell[11], and is necessary for the cyclical regeneration of the functionalis.

In addition to their differing function, there are histological differences between the functionalis and basalis layers. The functionalis comprises the outer two thirds and can be further divided into the most superficial layer consisting of few simple epithelial tubular glands in an abundant vascularised stroma (zona compacta), and the deeper zona spongiosa which has more glands but relatively sparse stroma[10]. The basalis consists of dense stroma, large blood vessels and glandular bases[10].

The endometrial epithelial glands are crucial to the normal physiology of the endometrium. The luminal epithelium is comprised of a single layer of columnar epithelium which invaginates into the underlying stroma to form the endometrial epithelial glands. The glands descend from the luminal epithelium through both the functionalis and basalis layers and to meet the myometrium[12]. In vitro recombinant tissue studies using mouse models demonstrated that the adult endometrial epithelium has the ability to grow in non-uterine stroma whilst maintaining its gene expression pattern[13]. In addition, the epithelial glands are the location of origin of the endometrial proliferative conditions. Proliferative conditions affecting the stroma, such as uterine stromal sarcomas, are very rare[14].

1.1.3 Stroma

The connective tissue surrounding the endometrial glands is known as the stroma. It has a vital role in the functioning of the endometrium, and there is much cross-talk between the glands and the stroma[15]. In addition to the complex extra-cellular matrix, it is composed of a variety of cell types. The primary cell-type is the stromal or reticular cell; a fibroblast-like cell which produces the extracellular matrix[10]. Other cell types present include natural killer cells and lymphocytes[16]. For all these cells, their morphology and density changes with both location in the endometrium and phase of the cycle. For example, lymphocytes represent just 7% of stromal cells during the proliferative cells, rising to 30% during early pregnancy[16]. The immunological complexities of implantation and pregnancy require a wide range of immuno-competent cells including T lymphocytes, macrophages and endometrial granulated lymphocytes[10]. Disruption in the numbers of the immune cells has been reported in various conditions including unexplained infertility supporting their role in the appropriate functioning of the endometrium[16].

1.1.4 Menstrual cycle

The menstrual cycle can be broadly divided into three phases (Figure 2); the proliferative phase (days 5-14), the secretory phase (days 14-28), and menstruation (days 1-4). The proliferative phase follows menstruation, during which in the absence of implantation, the functional layer of the endometrium is shed. This is triggered by a fall in both oestrogen and progesterone due to the destruction of the

corpus luteum. The effect of other ovarian steroid hormones with cyclical changes such as androgens on endometrium is not yet fully understood. The proliferative phase is marked by the regeneration of both the stromal and epithelial compartments of the functionalis. Some 3-4mm of endometrium grows during this phase under the influence of oestrogen. Once the endometrium is restored to its full thickness, the glands undergo significant changed in structure and secrete a glycogen-rich material ready to make the endometrium receptive to the blastocyst; these changes are the hallmark of the secretory phase.

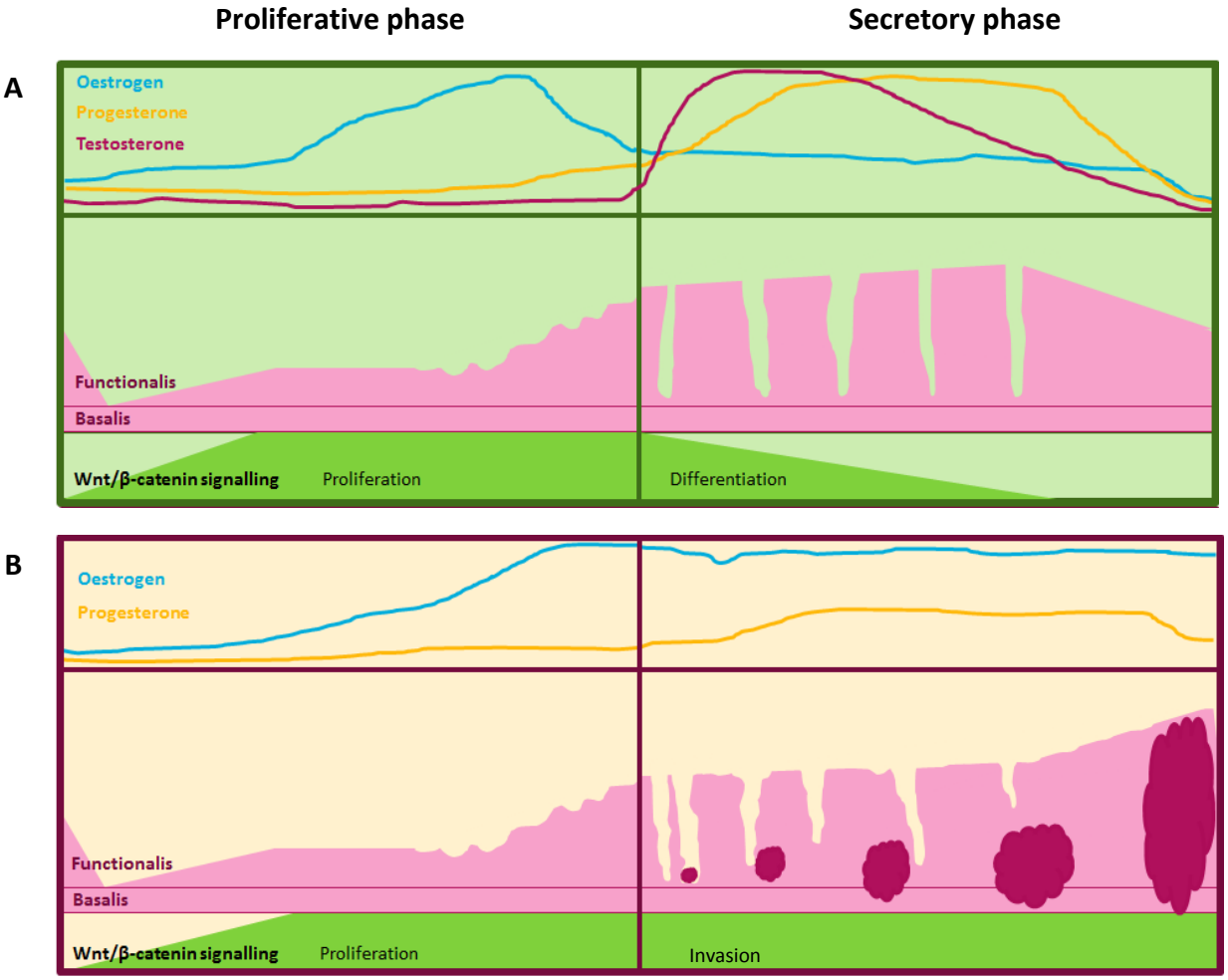


Figure 2: Hormonal and morphological changes during the normal menstrual cycle (A) and unopposed oestrogen exposure leads to proliferation and invasion of endometrial cancer (B).
Adapted from van der Horst *et al.*[3] and Cloke *et al.*[7].

1.1.5 Proliferative phase endometrium

The proliferative phase endometrium is characterised by the oestrogen-driven re-epithelisation following menstruation. As demonstrated in Figure 2, the proliferating endometrium is exposed to high levels of oestrogen with low circulating progesterone levels[3]. The endometrium is rapidly proliferating during this phase; thickness increases from approximately 1mm following menstruation to 4mm at the time of ovulation. The primary purpose of this phase is to re-assemble the cellular machinery necessary for the dynamic processes occurring later in the cycle[10].

The histology of the glands reflects the state of re-generation of the endometrium. The first glands to develop are characteristically straight and relatively undifferentiated. These become increasingly long and tortuous as the proliferation continues[10]. By the end of the proliferative phase, some degree of pseudostratification of the epithelium can be noted. The number of mitotic figures reflects the increasing levels of proliferation as the phase progresses.

The induction of proliferation by unopposed oestrogen exposure in the proliferative phase, is the closest that the benign endometrium comes to mimicking the conditions during endometrial carcinogenesis.

1.1.6 Hormone receptors

The steroid hormone receptors have a key role in the hormonal regulation of the endometrium. The actions of these receptors are mediated by both genomic and non-genomic signalling[10], resulting in differential expression across the layers of the endometrium, time during the cycle and levels of circulating hormones (Table 1).

1.1.6.1. Oestrogen receptor

Oestrogen is necessary for the essential processes of the normal functioning of the endometrium including proliferation and vascularisation[17]. Oestrogen receptor (ER) is expressed in both the epithelial glands and stroma of the endometrium. The two subtypes of ER α and β appear to have distinct functional roles[18]. ERα is known to up-regulate progesterone receptor and oestrogen-induced proliferation. ERα also has well-defined roles in endometrial carcinoma. The role of ERβ is far less clear[19], and it has been postulated that balanced co-expression of both the subtypes are an important factor in endometrial carcinogenesis[18]. In the normal endometrium, both ERα and ERβ are expressed in the in the epithelial glands and the stroma. Expression in the basalis does not appear to change during the menstrual cycle. However, expression in the epithelial glands in the functionalis layer decreased during the secretory phase, whilst stromal expression remained stable[20, 21].

1.1.6.2. Progesterone receptor

Progesterone receptor (PR) also has two subtypes (A and B)[22]. Expression is primarily regulated by oestrogen, resulting in PR induction in the proliferative phase[22]. This expression is down-regulated by its own ligand due to a negative-feedback loop. Consequently, expression of both subtypes is significantly reduced in the glands during the secretory phase, due to the dominant hormone being progesterone[23]. The basalis of the endometrium appears to be differently regulated, with PR being expressed by both the epithelial and stromal cells throughout the cycle[22]. The functional role of PR must be considered separately to its expression. It has been demonstrated using the Ishikawa cell line that glandular expression is not sufficient for a response to progesterone, and that stromal PR is a necessary condition[15].

1.1.6.3. Androgen receptor

Despite the lack of evidence currently available on the role of androgens in the endometrium[7], it is becoming increasingly apparent that together with oestrogen and progesterone, androgens may play an important role in the regulation of the menstrual cycle[22]. Androgen receptors are predominantly expressed in the stromal compartment of the endometrium[22]. The available literature suggests that a rise in AR corresponds with a fall in progesterone levels in the proliferative phase. This has been confirmed by *in vitro* studies using a well-differentiated cancer cell-line (Ishikawa) with primary stromal cells[24]. Whilst these experiments were not performed on normal cells, the Ishikawa cell line is the currently accepted surrogate

for understanding hormonal regulation in the endometrium, as there is not a benign cell-line available[25]. It would also appear that long-term progesterone treatment results in expression of AR in glandular epithelial cells[26]. Studies in non-human primate endometrium suggest that there is a complex interplay between the well-described oestrogen/progesterone mediated endometrial proliferation and differentiation and AR[27]. The androgen 5α-dihydrotestosterone (DHT) has been demonstrated to antagonise the proliferative effects of oestrogen, and there would appear to be considerable overlap in the genes which they regulate[28]. It has been postulated that up-regulation of AR in glandular epithelium is a key component in the anti-proliferative effects induced by progesterone[26, 29].

Table 1: Summary of the immunolocalisation of steroid hormone receptors in the functional layer of the human endometrium during the normal menstrual cycle. Adapted from Critchley *et al.* 2009[22].

Steroid receptor	**Glands**	**Stroma**	**Endothelium**
ERα	Positive, reduced during secretory phase	Positive	Negative
ERβ	Positive, reduced during secretory phase	Positive	Positive
PR	Positive, reduced during secretory phase	Positive	Negative
AR	Negative except after progesterone treatment.	Positive	Negative

1.1.7 Menopause

The endometrium is physiologically designed to respond to oestrogen and oestrogen-like molecules. In the absence of oestrogen, the endometrium becomes thin, atrophic, and unable to respond to progesterone[30]. Menopause, literally "cease in menstruation", results from ovarian failure, and is diagnosed in retrospect following 12 months of amenorrhoea. During the peri-menopausal period, ovarian function decreases creating an imbalance in the hypothalamic-pituitary-ovarian axis. A lack in circulating oestrogen results in the loss of the luteinizing hormone surge, which in turn causes prolonged follicular phases and anovulatory cycles[10].

During this phase, the histology of the endometrium reflects the hormonal status of the woman[10]. Once ovarian function has ceased, the post-menopausal endometrium still contains the luminal epithelium, stroma and glands. However, these glands are small and tubular, and the stroma resembles that of the pre-menopausal basalis[12]. Ultrasonic measurements of the thin post-menopausal endometrium range from 1-5mms in healthy women. This is in contrast to the 15mm thick secretory phase endometrium in young women. However, with the adequate hormonal supplementation, a fully functional endometrial functionalis layer can be regenerated from this thin post-menopausal endometrium[31] sufficient to carry a pregnancy to term[32].

Despite the atrophy that is the hallmark of the post-menopausal endometrium, nearly half of all endometria continue to show a weak proliferative pattern, either focal or diffuse, for many years following the cease of menstruation. This is probably

due to continuous low-level oestrogenic stimulation. Sivridis *et al.*.[33] outlined criteria for further sub-dividing the post-menopausal endometrium according to the proliferative profile.

- **Atrophic and inactive endometria:** endometrium is deprived of a functionalis layer and consists exclusively of a thin basalis with a few narrow tubular glands, lined by cuboidal indeterminate epithelium, showing neither proliferative nor secretory activity.

  A frequent variant of this structure included tubular atrophy. An atrophic endometrium showed cystically dilated glands, lined by flattened indeterminate type epithelium.

- **Atrophic/weakly proliferative endometria:**
  - Shallow endometrium 2.2mm thick with loss of distinction between the basal and functional layer.
  - Proliferative type tortuous endometrial glands. Tall columnar pseudostratified epithelium, oval nuclei, and very infrequent mitoses.
  - Dense, fibrotic endometrial stroma, devoid of mitoses.

- **Mixed:** largely atrophic and inactive endometria which show focal areas of weakly proliferative glands.

The majority of cancers are related to endometrial atrophy, which in turn originate from weakly proliferative glands. Aromatase catalyses the peripheral aromatisation

of adrenal androstenedione to oestrone[34]. This process increases with body weight and age due to increased specific activity of aromatase. These factors appear to be additive in their effect; both factors are often present in patients. Despite being less potent than oestrogen, oestrone is the single most important oestrogenic compound in endometrial carcinogenesis.

In the absence of an operational ER system, oestrogenic stimulation would be ineffective. The post-menopausal endometrium retains its low potential for carcinogenesis via the weak proliferative activity seen in many glands. 86% of non-malignant tissue without neoplastic features adjacent to endometrial carcinomas demonstrated a focally proliferative pattern[33]. It is likely that the minority which did not display proliferative foci, were not oestrogen independent, but rather that the proliferative foci had been obliterated by the invading tumours.

1.1.7.1. Androgen in the post-menopausal endometrium

Following the cessation of ovarian activity, circulating blood and endometrial levels of *estradiol* dramatically decrease, resulting in the onset of menopausal symptoms (Figure 3). Residual circulating oestrogen levels are maintained by extra-ovarian conversion[6]; cytochrome p450 aromatase catalyses the peripheral aromatisation of adrenal androstenedione to oestrone. Androgen (androstendione and testosterone) levels only slightly reduce during this period, and are converted to oestrones. There is conflicting evidence on whether the adrenal glands or the ovaries are the main

source of circulating androgens in the post-menopausal endometrium; it is likely that both sites contribute to overall levels[6].

Endometrial adenocarcinoma is a disease of the post-menopausal endometrium, and since androgens are the dominant hormone following ovarian failure, it has been postulated that they have a role in endometrial carcinogenesis[35]. Androgens have been proposed to have a direct, AR-mediated impact in addition to an indirect, ER mediated impact on endometrial proliferation and inflammation[35].

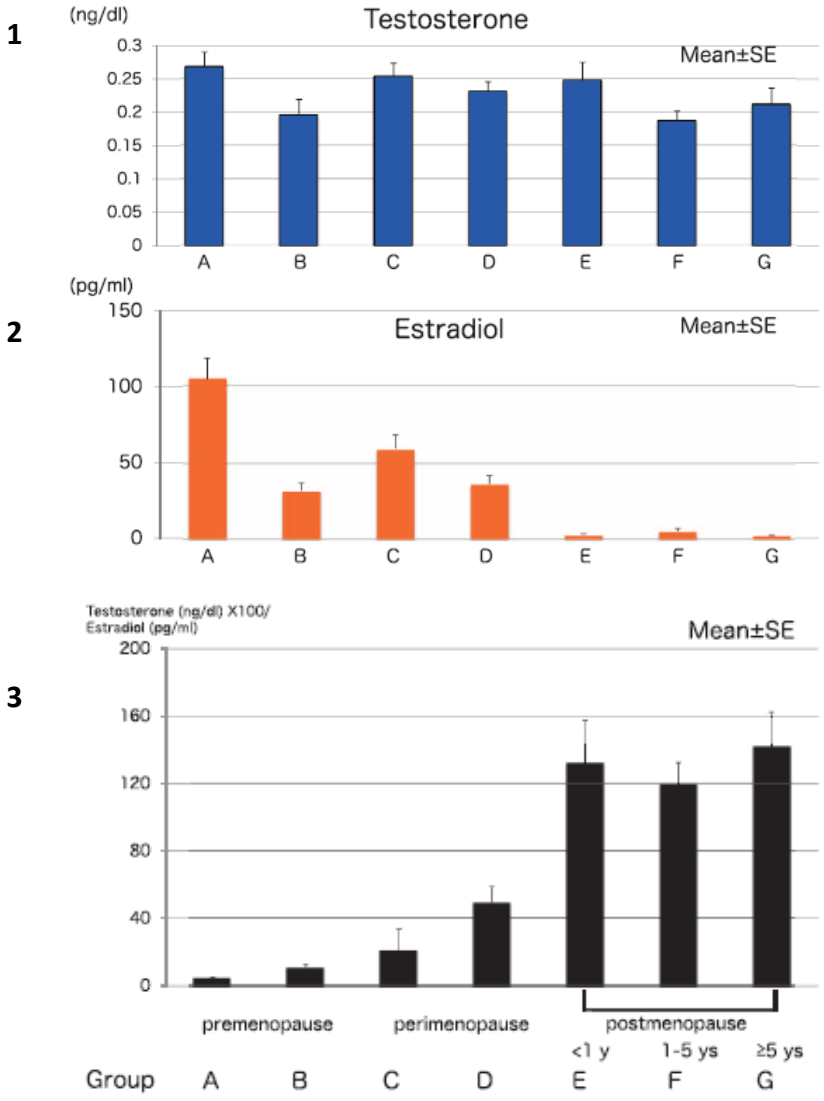


Figure 3: Testosterone in the menopause. Changes in levels of total testosterone (1), total estradiol (2) and their relative ratio (3) during the menopausal transition. A) Early reproductive stage. B) Late reproductive stage. C) Early menopausal transition. D) Late menopausal transition. E) Within 1 year of menopause. F) Within 5 years of menopause. G) 5+ years since menopause. Adapted from Yasui *et al.* 2012[6]

1.2. Endometrial carcinoma

Endometrial cancer can be broadly divided into two categories; type 1 and 2[4]. Bokhman *et al.*. first designated these types because they show significant demographic, pathological and clinical differences. The majority of all endometrial carcinoma cases (approximately 80%), are the oestrogen dependent type 1 endometrioid adenocarcinomas.

Type 1	**Type 2**
– Endometrioid adenocarcinoma	– Papillary serous adenocarcinoma
– With squamous differentiation	– Clear cell adenocarcinoma
– Villo-glandular	– Mucinous adenocarcinoma
– Secretory	– Undifferentiated carcinoma
– With ciliated cells.	– Mixed carcinoma

1.2.1 Epidemiology

Endometrial carcinoma is the most common gynaecological cancer, and the fourth most common cancer in women. With a lifetime risk of 1 in 46[36], it is almost twice as common as ovarian cancer. This is primarily a disease of post-menopausal women, with 93% of cases being diagnosed in women over the age of 50, with a peak incidence of 83 per 100,000 in women in their early 70s. The epidemiology of this disease has varied greatly over the past forty years, with large variations in both incidence and mortality.

From the late 1970s onwards the mortality rates plummeted by over a third, only to rise again by a fifth during the new millennium to become the 9th most common cause of cancer death among women[37]. Similarly the age-standardised mortality rates remained virtually stable until the late 1990s, but have since increased by over 40%. Currently there are approximately 8000 new cases a year in the UK which accounts for almost 2000 deaths, but this is set to increase. It would appear that the improvements seen due to surgical improvements have been counteracted by the continual rise in incidence and lack of effective non-surgical options. In fact the 5-year survival rates have remained stable in recent years 1994-2004[38], despite the significant improvements seen in other malignancies.

Multiple studies have pointed to a concerning rise in incidence. An age-period-cohort analysis of epidemiological data in Norway have predicted that incidence rates of endometrial cancer will increase by up to 100% between 2007-2025[39]. It has even been reported that in 2015, the incidence will be twice those of 2005[38]. This concerning level of increasing incidence across the developed world, is primarily attributable to increasing life expectancy coupled with the insidious continual rise in body mass index (BMI)[39].

1.2.2 Risk factors[4,40]

The primary risk factors for type 1 endometrial cancer are those associated with excessive exposure to oestrogen. The key risk factors include increasing age, obesity, early menarche and late menopause as well as low parity.

- Polycystic ovarian syndrome
- Family history
- Lynch syndrome
- Oestrogen secreting tumours
- Diabetes mellitus
- Hypertension
- History of breast cancer
- Immunodeficiency
- Unopposed oestrogen only hormone replacement therapy
- Tamoxifen therapy
- Dietary factors
- Previous radiotherapy

Protective factors include cigarette smoking, combined oral contraception for at least one year, and grand multiparity.

Obesity is the single most important independent risk factor for endometrial cancer for a number of reasons, delivering a 3.5-fold increased risk[41]. In fact it is estimated that 70-90% of women diagnosed with endometrial cancers are overweight, and a growing number of these cases are attributed to obesity[41]. In pre-menopausal women, obesity can disrupt the normal menstrual cycle by causing insulin resistance, ovarian androgen excess and chronic progesterone deficiency[42]. In post-menopausal women, circulating weak oestrogenic compound oestrone is derived from the conversion of androgens of both adrenal and androgen origin to oestrogen

by extra-ovarian aromatisation in peripheral adipose tissues; in obese women this can cause a hyper-oestrogenic state. These high levels of oestrogen unopposed by progesterone, via oestrogen receptors, induce endometrial cell proliferation, therefore increase the likelihood of mutations[41].

1.2.3 Clinical features[4]

Classically the hallmark of endometrial carcinoma is post-menopausal bleeding; in pre- and peri-menopausal women, this may present as intermenstrual/irregular bleeding. Other clinical features include pain, vaginal discharge, and pyometra, which can be associated with advanced disease. Early detection by awareness of symptoms is crucial for good management and prognosis. Survival for early stage disease is very good; however, this drops to 45% 5-year survival in Stage III and 25% in Stage IV[43]. Consequently, all patients presenting with post-menopausal bleeding should have a transvaginal ultrasound scan and endometrial biopsy as a minimum; the gold standard is hysteroscopy and biopsy[44] (Figure 4). Definitive diagnosis of endometrial cancer is histological.

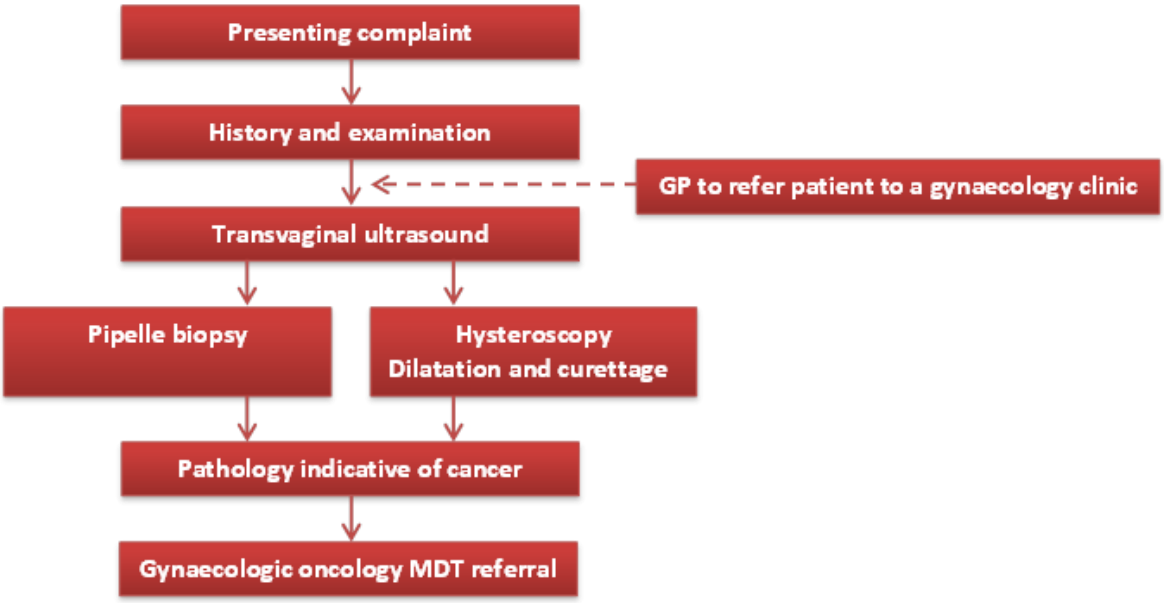


Figure 4: NICE guidelines for the diagnosis of endometrial adenocarcinoma. Adapted from Saso *et al.*[4].

1.2.4 Pathology and staging

The International Federation of Gynaecology and Obstetrics (FIGO) classification of grading and staging in endometrial cancer is currently the most widely accepted method,[45] and remains the most useful prognostic guide (Table 2 and 3). Low and high grade cancers can be distinguished by the percentage of solid or non-glandular areas, pattern of invasion and the presence or absence of tumour necrosis[43]. The level of differentiation with the cancer cells is an important prognostic factor. Since well differentiated cells usually maintain their steroid receptors, and therefore their receptivity to hormones, it is not surprising that poorly differentiated tumours are associated with deep myometrial invasion and metastasis[46].

Table 2: FIGO descriptors of grading of endometrial adenocarcinoma. Adapted from Saso *et al.* [4].

FIGO Grade	Description
Grade 1	<5% of solid/non-glandular areas
Grade 2	6-50% of solid/non-glandular areas
Grade 3	>50% of solid/non-glandular areas

Staging is performed surgically, based on the operative findings at hysterectomy.

The criteria are outlined below.

Table 3: FIGO description of staging in endometrial adenocarcinoma. Adapted from Saso *et al.* 2011[4]

FIGO stage	Description
Stage 1	Tumour confined to the corpus uteri
Stage 1A	No or less than half myometrial invasion
Stage 1B	Invasion equal to or more than half of the myometrium
Stage 2	Tumour invades cervical stroma but does not extend beyond the uterus
Stage 3	Local and/or regional spread of the tumour
Stage 3A	Tumour invades the serosa of the corpus uteri and/or adnexae
Stage 3B	Vaginal and/or parametrial involvement
Stage 3C	Metastases to pelvic and/or para-aortic lymph nodes
Stage 3C1	Positive pelvic nodes
Stage 3C2	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
Stage 4	Tumour invades bladder and/or bowel mucosa, and/or distant metastases
Stage 4A	Tumour invasion of bladder and/or bowel mucosa
Stage 4B	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

The distant spread of disease from the uterine cavity to other tissues either by invasion or metastasis is the main cause of death in endometrial cancer. In endometrial cancer, spread is primarily by local invasion to the myometrium, fallopian tubes, cervix, vagina, bladder and rectum. Lymphatic spread to the pelvic, para-aortic and inguinal lymph glands is also common. Despite recent advances in treatment and patient care advanced stage disease has a very poor prognosis[43, 46, 47] (Table 4).

Table 4: Proportion of cases diagnosed by stage, and the 5-year survival rates by stage. Adapted from Bradford *et al.* 2013[47]

	Stage at presentation (%)	5-year survival rate (%)
Stage I	73	85-90
Stage II	11	70
Stage III	13	40-50
Stage IV	3	15-20

1.2.5 Management

The management of endometrial cancer is primarily based on the stage at which it is diagnosed (Figure 5). Primarily the management is surgical; total abdominal hysterectomy with bilateral salpingo-oophrectomy, with peritoneal washings[47]. In advanced disease, pelvic and para-aortic lymphadenectomy and omentectomy can also be performed[42].

The use of adjuvant therapy in localised endometrial cancer has been widely discussed, and no consensus has been achieved. The role of radiotherapy in

endometrial cancer remains unclear[48], but is frequently used in advanced disease especially in the palliative setting.

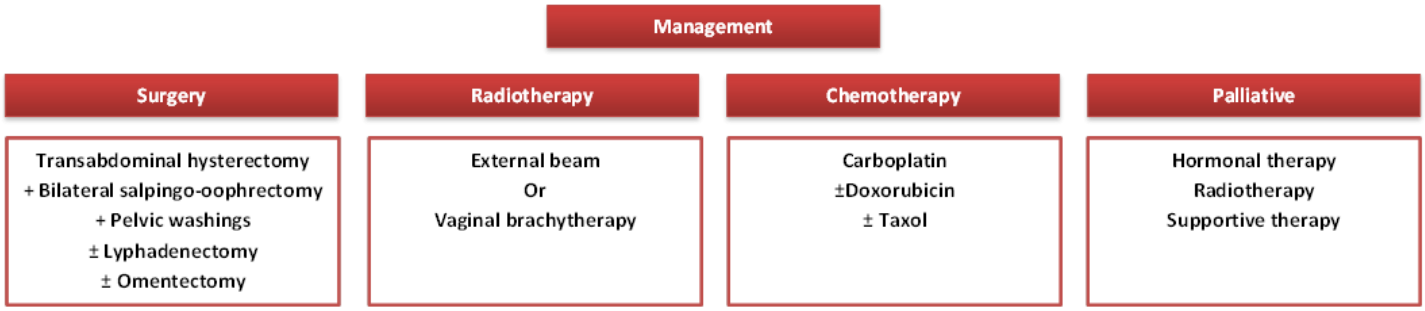


Figure 5: Management options for endometrial adenocarcinoma. Adapted from Saso *et al.*[4].

1.2.6 Model of pathogenesis

Carcinogenesis in the endometrium requires the accumulation of multiple molecular changes in common with all cancers. The precise mechanisms of how known risk factors, hormonal imbalance for example, contribute to carcinogenesis is poorly understood[40]. The characteristic feature of endometrial carcinogenesis is disruption of the glandular architecture, followed by glandular crowding and loss of peri-glandular stroma[10]. These histological features disrupt normal homeostasis by altering the paracrine mechanisms, as the stroma plays a key role in hormonal regulations[15].

The single most important risk factor for Type 1 carcinomas is prolonged oestrogen exposure, with 40% of cancers being directly attributed to obesity[40]. During a normally regulated cycle, progesterone provides a counter-balance to high oestrogen levels during the secretory phase. If there is an imbalance in this system, either due to low progesterone levels or inappropriately high oestrogen levels, the

oestrogen mediated proliferative effect becomes dominant leading to endometrial hyperplasia. In fact progesterone appears to inhibit epithelial to mesenchymal cell transition; loss of progesterone signalling (which frequently occurs in progressive disease) releases the inhibition, and permits tumour progression[49]. Endometrial adenocarcinoma does have a related pre-malignant hyperplastic stage, but is certainly not present in all cases[33]. Whilst it certainly holds an important role, oestrogen alone is not sufficient for tumorigenesis – somatic mutations are also required[40].

The genetic mutations required for carcinogenesis are accumulative, with no single mutation being sufficient alone. In the endometrium, common genetic mutations include adenomatous polyposis coli (APC), phosphatase and tensin homologue (PTEN) and β-catenin[50]. The DNA mis-match repair genes (MLH1, MSH2, MSH) are also often implicated,[40] as are tumour protein 53 (TP53) and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS).[51]

Possibly the single most important feature of endometrial carcinogenesis is aberrations in the canonical Wnt/β-catenin pathway, which may be inappropriately induced by excess oestrogen as would happen during the proliferative phase of the normal menstrual cycle.

1.3. Wnt-related proteins

1.3.1 The canonical Wnt/β-catenin pathway

The canonical Wnt/β-catenin pathway has long been known to be a key regulator in development, disease and homeostasis. There are three pathways believed to be activated by Wnt, of which the canonical pathway is the best understood[52]. The canonical Wnt pathway promotes cell proliferation and tissue expansion, controlling fate determination and terminal differentiation[52].

The cytoplasmic protein β-catenin lies at the centre of the canonical Wnt/β-catenin cascade (Figure 6). In the absence of Wnt activation, β-catenin is bound by the destruction complex which is composed of the tumour suppressors adenomatous polyposis coli (APC) and axin. The kinases CK1 and GSK1 from the destruction complex then phosphorylate β-catenin, which is then targeted for proteosomal degradation[1]. On the other hand, if Wnt receptors are occupied, the kinase activity of the destruction complex is inhibited, allowing β-catenin to accumulate and travel to the nucleus where it binds to the N-terminus of the DNA-binding proteins of the Tcf/Lef family[1]. β-catenin converts Tcf/Lef factors into transcriptional activators, albeit transiently; this facilitates the transcription of the down-stream Wnt genes. Whilst it is accepted that once stabilised by Wnt signals, β-catenin translocates to the nucleus in order to reprogram the responding cell, there is no consensus on the mechanisms by which this occurs[52]. In some cases, cells undergoing Wnt signalling show an increased β-catenin expression, rather than a nuclear preference[52].

In addition to its signalling properties, β-catenin is also a key component of the adherens junctions of simple epithelial cells[52, 53]. It remains unclear whether the adhesive and signalling properties are inter-connected; in other organisms these functions are performed by different homologs[52]. It is hypothesized that when first synthesized, β-catenin is made available to the adhesions junction. When this pool is saturated, the free cytoplasmic β-catenin is degraded by the destruction complex; only this highly unstable pool is regulated by the Wnt signals[52].

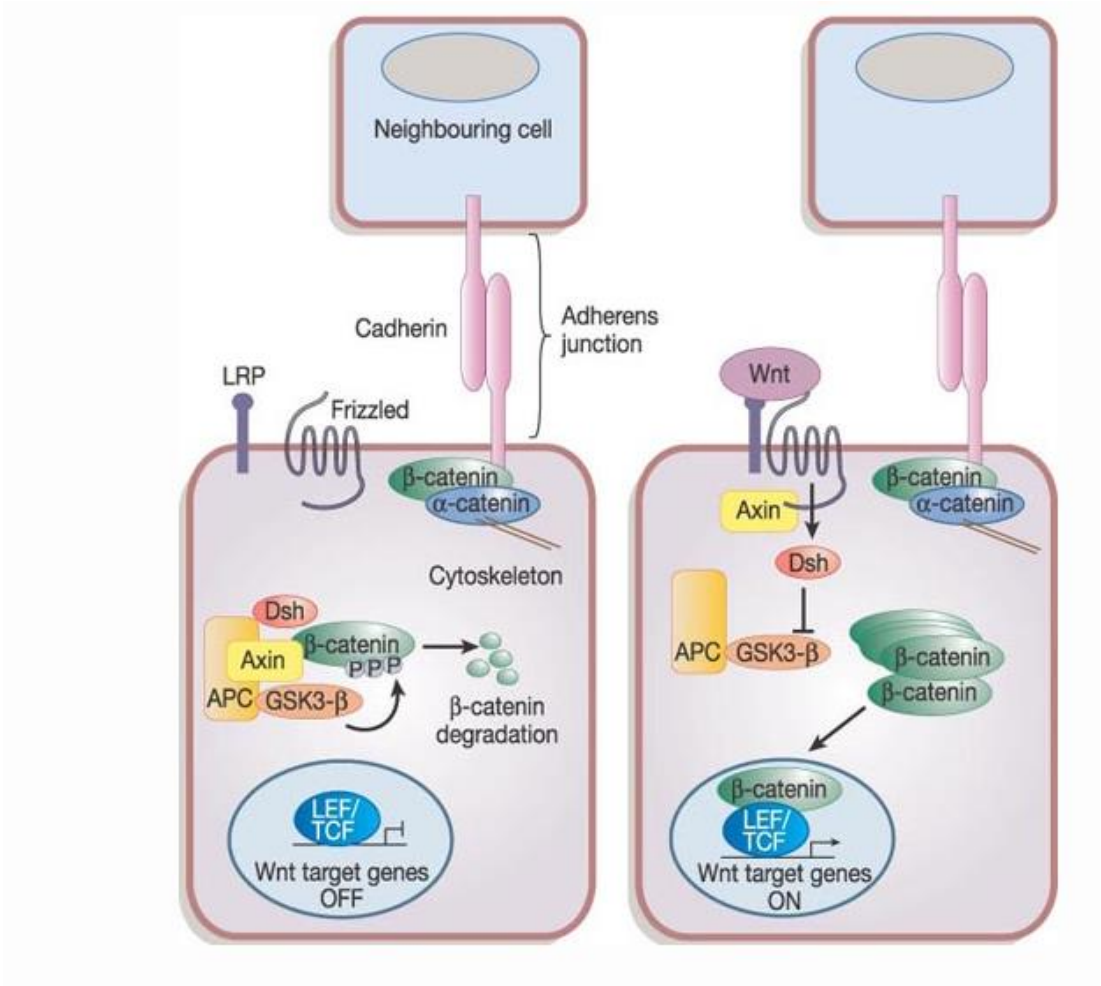


Figure 6: The canonical Wnt/β-catenin pathway. Left panel: In the absence of Wnt signalling, β-catenin is bound to the destruction complex which leads to its degradation. β-catenin also binds to cadherins and regulates cell-cell adhesion. Right panel: In the presence of Wnt signalling, β-catenin translocates to the nucleus, where it binds to Lef/Tcf transcription factors, and causes tramscription of downstream proliferative genes. From Clevers *et al.*[5]

1.3.2 Role in self-renewing tissues

In many self-renewing tissues, such as the gut, hair follicles, haemopoietic system, bone and endometrium, the Wnt pathway remains vital after development and continues to be essential throughout life[52]. Wnt signalling has been shown to be involved in regulating progenitor cell populations in diverse tissues, including the intestine[5].

The intestinal and endometrial epithelium share striking similarities[12]. They are both dynamic, highly proliferative tissues that rapidly renew. The intestinal epithelium is arranged along the crypt-villus axis in a monolayer[5]. Like the basalis of the endometrial glands, the crypts of Lieberkuhn contain the stem/progenitor cells (Figure 7). These produce daughter cells which migrate upwards from the crypt towards the villus tip and differentiate into the differentiated cells; enterocytes, goblet and enteroendocrine cells[54]. In the gut Wnt proteins are expressed by the epithelial glands, but not the surrounding mesenchyme; they promote the proliferation of the progenitor cells as well as promoting terminal differentiation of the Paneth cells[52]. It is currently thought that the Wnt pathway is the single most important regulator of cell fate in the crypt-villus axis[5]. In another similarity between the two epithelial tissues, the stem-like progenitor cells of the intestine also reside in the base of the glands[5].

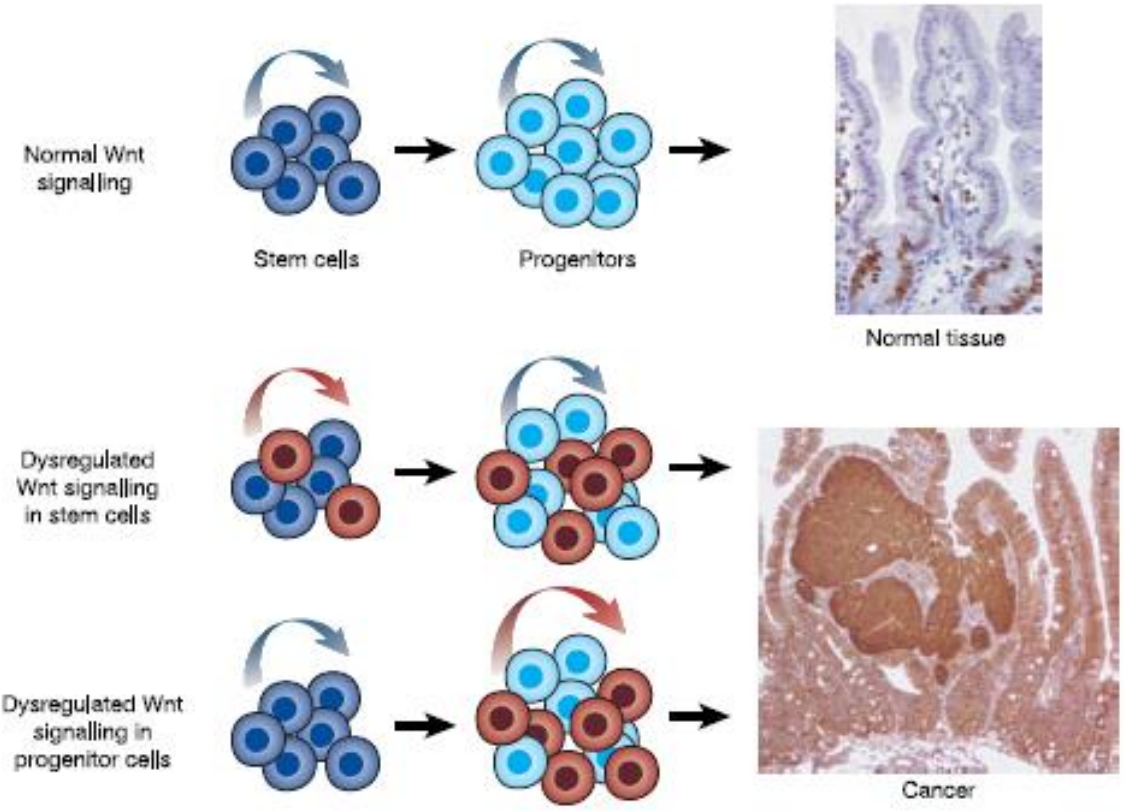


Figure 7: Intestinal progenitor cells reside in the base of intestinal crypts. Normal Wnt signalling influences the proliferation and renewal of stem and progenitor cells. Uncontrolled Wnt signalling (red arrow) leads to constitutive renewal and aberrant expansion of the stem cell pool, or confer stem cell behaviour pm the progenitor cell pool. These changes can lead to the formation of cancerous tissues (cancerous cells are denoted in red). From Reya *et al.*[1]

1.3.3 Endometrial physiology

The development of the Müllerian duct is dependent on correct functioning of the Wnt pathway; in the absence of Wnt proteins including Wnt4, 9b, 5a and 7a, development will cease at key times[3].

A functional canonical Wnt/β-catenin pathway has recently been demonstrated, in both the pre- and post-menopausal normal endometrium[32]. It is hypothesized that oestrogens induce Wnt/β-catenin expression during the proliferative phase, and progestagens inhibit it during the secretory phase, providing a counter-balance[3]. This is supported by β-catenin expression. Nuclear staining is seen in the

proliferative phase, but mainly cytoplasmic and junctional staining are seen during the secretory phase[50]. This phasic change in localisation would suggest a role for the sex steroid hormones in controlling Wnt/β-catenin staining[50]; an interaction has been demonstrated in an *in vivo* model [55], but further work is required.

The putative endometrial stem/progenitor cell is thought to be located in the basalis of the pre-menopausal endometrium, and is therefore retained through both menstruation and the menopause[32]. Differential expression of BMP, NOTCH, Hedgehog, and FGF signalling pathways in conjunction with Wnt-related genes suggest cross-talk and the possible existence of stem cell signalling networks in a similar fashion to that found in the intestine[32]. One such interaction may involve telomerase, which is thought to play a key role in the endometrium[56], but has also been suggested to be a co-factor of β-catenin in order to prevent the undermining of Wnt-regulation[57].

1.3.3.1. Ovarian steroid hormones and the canonical Wnt/β-catenin pathway

Hormonal regulation through the hypothalamic-pituitary-ovarian axis has long been known to be vital to regulation of the menstrual cycle, via oestrogen-induced proliferation and progesterone-induced differentiation[58]. The signalling pathways through which this is mediated are far less well understood.

Endometrial proliferation appears to be regulated by a combination of oestrogen stimulation and Wnt signalling. Estradiol can induce β-catenin stabilisation, and therefore transcription of down-stream proliferative genes. Inhibition of Wnt

signalling also results in the abolition of estradiol-induced proliferation[40]. These two mechanisms are inextricably linked, with ERα acting as a transcription factor for the Wnt ligands Wnt4, Wnt5a, and Wnt7a. Estradiol appears to be able to affect the transcription of Wnt target genes without directly interacting with oestrogen response elements at the DNA level[59].

Progesterone can act as a profound inhibitor of Wnt/β-catenin signalling[60]. This may be a result of induction of DKK1 and FOXO1, both of which are important Wnt inhibitors[61]. Wnt/β-catenin may also act down-stream to the Hedgehog signalling pathway which is sex steroid hormone regulated; down-regulated by progesterone, and up-regulated by oestrogen[40, 59]. It is hypothesized that inhibition of the Wnt pathway by progesterone is an important anti-progression mechanism in endometrial cancer[49]. Loss of progesterone signalling leads to enhanced Wnt/β-catenin signalling in progressive disease[3].

The relationship between androgens and the Wnt/β-catenin pathway have been extensively studied in some tissues; this does not extend to the endometrium where there is scant evidence[62]. Androgen receptor and the Wnt/β-catenin pathway appears to interact during progression of prostate cancer[63], although this may not be the case in benign tissue. AR and β-catenin are dynamically linked; β-catenin can perform as a co-activator of AR, and AR may be involved in nuclear co-trafficking and transrepression of β-catenin in a variety of tissue types including prostate, colon and neurones[64].

1.3.4 Role in cancer

Despite being essential to life in some tissues, mutations along the Wnt pathway can disrupt homeostasis and cause pathologies including cancer[52]. The demonstration of an interaction between β-catenin and APC, was the first link between human cancer and the Wnt/β-catenin[52]. In colon cancer, loss of APC is one of the first mutations to occur which results in constitutive activation of the Wnt pathway, as the destruction complex can no longer function.

The evidence of a role for Wnt in endometrial adenocarcinoma is growing, and it is likely that Wnt activation is likely to play a role in carcinogenesis. The Wnt pathway has many self-regulating aspects, as many Wnt-downstream proteins have positive and negative regulating properties[52]. Many members of the pathway can accumulate loss- and gain-of-function mutations, which among others has lead APC to be identified as a tumour suppressor, and β-catenin as an oncogene[40]. Approximately 40% of well-differentiated endometrial adenocarcinomas demonstrate nuclear β-catenin staining suggestive of an activated Wnt pathway[3]. One contributory factor is the frequency of APC mutations, which has been reported to be as high as 43%[51]. In addition to its role in the destruction complex, APC regulates other essential processes including cell proliferation, death, differentiation, migration and adhesion[51]. In addition, activating mutations in β-catenin itself have been reported in 15-40% of endometrial tumours[65, 66].

Mouse models have shown that activation of the Wnt pathway can induce endometrial hyperplasia and affect oestrogen signalling[51]. Hormonal signalling

pathways require correct epithelial-stromal cross-talk, in which Wnt plays an important role. However, Wnt activation alone was not sufficient for full malignant transformation.

1.3.5 SOX9

The sex-determining region Y box (SOX) factors are a large family of transcription factors that share a homologous high-mobility group DNA-binding domain[67]. This family is divided into 8 groups (A-H), of which SOX9 belongs to group E. SOX9 is known to have many important and diverse developmental roles including chondrogenesis, male gonads, neural crest and spinal cord glial cells[67]. SOX9 is capable of causing diverse disease states due to its roles in controlling the pericellular environment, cell differentiation and proliferation. The molecular mechanisms of this range from the transcription level to post-translational modification, nuclear transport and co-factor interaction[68].

SOX9 is also important in the maintenance of tissues, most notably for its regulatory role in the canonical Wnt/β-catenin pathway. In the intestine, SOX9 is expressed in the stem/progenitor cells of the rapidly proliferating intestinal crypts, and is required for Paneth cell differentiation[68-70]**.** It is also likely that SOX9 may regulate the number of stem cells in the intestinal epithelium; SOX9 knock-down mice express more Musashi-expressing cells[70].

1.3.5.1. SOX9 is a modulator of the Wnt/β-catenin pathway

The SOX family of transcription factors are increasingly being recognised as key modulators of the canonical Wnt/β-catenin pathway. The expression of SOX9 and Wnt proteins are inextricably linked, with SOX9 expression in the mouse intestine being dependent on Wnt signalling[70]. The expression pattern of SOX9 is suggestive of a complex transcriptional regulation, and there have been a diverse range of Wnt associations described in the literature. SOX9 has been described as both a Wnt transcriptional target and a key canonical Wnt pathway regulator[69].

In the intestine, the expression of SOX9 mirrors the domain of epithelial cells stimulated by Wnt molecules. The TCF4-β-catenin complex is necessary for the expression of SOX9, which has been confirmed in knock-out studies using TCF4-null mice[71]. However, SOX9 subsequently locally attenuates the expression of Wnt-target genes, thus regulating Wnt-stimulated proliferation[70]. SOX9 has therefore been proposed as a gatekeeper of canonical Wnt-pathway activation, although several mechanisms have been described. SOX9 has been demonstrated to down-regulate[70], and in some studies to, inhibit[72] β-catenin/TCF-dependent transcription[70]. In addition, SOX9 both inhibits the activation of the β-catenin-dependent promoters and stimulates β-catenin degradation[73]. In chondrocytes, up-regulated SOX9 produces the same phenotype as a loss of β-catenin function. Conversely, loss of SOX9 expression resembles constitutively activated β-catenin. Regardless of the exact mechanism, a classical negative feedback loop is well described in the murine intestinal epithelium[69], and ensures that Wnt-stimulated

proliferation is kept in check[70, 71]. Aberrations in this closely controlled relationship have been implicated in the pathogenesis of several cancers.

1.3.5.2. SOX9 is implicated in carcinogenesis

SOX9 expression has been reported in various human malignancies, including lung adenomas and prostate carcinomas[67]. The possible significance of this has not yet been fully determined, and it would appear that SOX9 has differing roles between types of malignancy[67]. One of its roles in carcinogenesis may be involvement in epithelial-to-mesenchymal transition (EMT). EMT is one of the processes which permit metastasis, due to previously immobile epithelial cells gaining mesenchymal characteristics[74]. SOX9 is known to be involved in EMT during normal development, but is also speculated to be involved in EMT in both prostate and colon cancer[68].

The currently available data propose opposing roles for SOX9 in malignancy. For example, up-regulation in lung adenomas results in gain of tumour growth potential and tumorigenicity[67]. However, in prostate carcinoma elevated expression results in a decreased rate of cellular proliferation, cell cycle arrest in grade 0/1 cancers and an increased sensitivity to apoptosis[67]. Moreover, SOX9 has been proposed to both induce and inhibit cell proliferation. It is necessary to consider possible interactions with signalling pathways in order to fully understand the implications of SOX9 expression in malignancy[68]. During normal chondrogenesis, SOX9 is regulated by Wnt, TGF-β, Notch and FGF all of which have been investigated in relation to malignancy.

1.3.5.3. The expression of SOX9 in the endometrium

The role of SOX9 in the endometrium is largely undetermined, but it has been suggested that its expression may play a key role in the establishment and maintenance of characteristic phenotypes of glandular cells during the menstrual cycle[67]. Saegusa *et al.* have reported nuclear SOX9 staining in a subset of epithelial, but not stromal endometrial cells. They described an increase from normal, to hyperplastic, to malignant samples, with a similar correlation between increasing grade[67]. There also appears to be a bi-phasic up-regulation during the proliferative and late-secretory phases of the normal endometrial cycle[12, 67]. However, a high level expression of SOX9 in the post-menopausal endometrium has been subsequently reported[12], which was not investigated by Saegusa *et al.*. The emerging role for SOX9 in keeping proliferation under tight regulatory control, thereby suppressing Wnt mediated proliferation would support a high SOX9 expression in the largely atrophic post-menopausal endometrium[12]. In addition, SOX9 appears to be down-regulated by oestrogen, and therefore an elevated expression in the hypo-oestrogenic could be expected[75]. SOX9 may consequently act to prevent the development of hyperplasia, as loss of intestinal SOX9 leads to hyperplasia[70]. The relative expression between post-menopausal and malignant endometrium remains unknown.

1.3.6 NAP1L1

Nucleosome assembly protein 1-like 1 (NAP1L1), is a nuclear protein involved in modulating chromatin formation and contributes to the regulation of cell proliferation. To date the exact function of NAP1L1 is not clear although it is speculated to be involved in regulating DNA replication, gene expression, and cell growth[76]. NAP1L1 is the human equivalent to the yeast NAP1 gene[77], whose roles include; histone chaperone, chromatin-assembly factor, role in tissue-specific transcription regulation, histone shuttling, apoptosis and cell-cycle regulation[78]. Increased expression of NAP1L1 may be related to the progression of cell growth, as both the mRNA and protein levels increases with the induction of cell proliferation in a T-lymphoid cell model[77].

Chromatin assembly is a vital process in the maintenance of genomic integrity[79], the histone chaperone proteins such as NAP1L1 have 3 primary roles. Firstly to regulate chromatin assembly, secondly to mediate chromatin disassembly during transcription and replication, and thirdly to create specialised chromatin structures containing histone variants[79].

NAP1L1 has been discussed in relation to stem-like cells in the heart. It is suggested that down-regulation of NAP1L1 may facilitate the transition from stem cells into cardiomyocytes[78]. NAP1L1 has frequently been described as a cancer-related protein[78], showing up-regulation in both hepatoblastomas[80] and breast cancer[81]. Studies have shown that the NAP1L1 gene can be used to identify, both the primary

hepatoblastoma tumour, but also their metastases[77]. The evidence in colon cancer is similarly promising, with approximately 50% of cases showing over-expression. NAP1L1 could be considered to be a proliferation marker of colon cancer cells, and theoretically could be investigated as a future therapeutic target[82]. Moreover in breast cancer, NAP1L1 has been studied as a potential marker of resistance to chemotherapeutic agents. Over-expression of NAP1L1, together with NAP1L4, exerts a protective effect against doxorubicin-induced cytotoxicity[81]. NAP1L1 has also been identified to induce 4-hydroxytamoxifen in the breast cancer cell line T37D[81]. There have not been any published reports of NAP1L1 in either the normal or malignant endometrium.

1.3.7 SFRS2

Serine/arginine-rich splicing factor 2 (SFRS2) also known as splicing factor 35 (SC35), is one of the 8 classical[83] serine/arginine-rich (SR) splicing factors. The SR splicing factors are an essential component of the spliceosome, which acts to remove introns from precursor mRNA. They allow alternative splicing, a fundamental process controlling gene expression in differentiation and development[84]. The result of alternative splicing of pre-mRNA is to generate functionally diverse isoforms from a single gene, enabling cell and tissue specific gene expression in response to both developmental and environmental cues[85]**.** Whilst this is usually a regulated step in the expression of eukaryotic genes, it can become dysregulated in carcinogenesis, with many cancer-associated genes being regulated by this process. Furthermore, many splice variants (created by the selective joining of different

exons) are unique to the malignant state. Whilst the exact role that these variants play in the development and maintenance of a tumour is yet to be established, it can be hypothesized that they have potential as diagnostic indicators and therapeutic targets[85].

SFRS2 is increasingly being investigated in a variety of malignancies including cervical, ovarian, breast and colon, all of which have similarities with endometrial cancer. There are a number of postulated roles and mechanisms; there is a marked induction of SFRS2 in ovarian cancer relative to normal ovarian tissue[85]. Pharmacogenomics studies have identified SFRS2 to be among a group of molecular determinants of sensitivity and resistance to chemotherapeutic agents including artesunate[86], which can be used to treat both breast and colon cancers. Expression of SR family members, including SFRS2, has been shown to be altered during mammary tumorigenesis, leading to alterations in the normal splicing of a number of pre-mRNA[87].

SFRS2 is required for the alternative splicing of KLF6, a tumour suppressor gene[85], which has an important role in breast, prostate and colon cancers. This has led to speculation as to whether SFRS2 is a proto-oncogene, and its ability to regulate the genes identified by Shi *et al.*. gives it its transforming ability[85].

SFRS2 showed abundant nuclear staining in cervical intraepithelial neoplasia (CIN) 3, with a trend of increasing over-expression throughout the grades of CIN[83]. The results might suggest a role for SFRS2 in cervical tumour formation. One promising

avenue of investigation is the use of SR proteins for diagnosis of cervical tumour progression; SFRS2 marks the nuclei of rapidly dividing basal epithelial cells. In high-grade cervical lesions, these basal cells fill the full thickness of the epithelium, giving a uniform staining.

Epithelial cancer cells surfaces are covered by complex carbohydrates[88], which function in both malignancy and metastasis. Whilst no mechanism has been defined, it is postulated that there is a correlation between cancer-associated carbohydrate antigens and poor patient prognosis, including metastasis. Hatakeyama *et al.* suggest that the SFRS proteins are expressed by a subset of lung capillaries, and it is these which are responsible for the carbohydrate-dependent cancer cell colonization of the lung in the mouse. However, they cannot exclude that it is another carbohydrate-binding receptor which mediates this mechanism.

Merdzhanova[89,90] *et al.* investigated whether E2F1 and its direct transcriptional target SFRS2 could control VEGF-A expression and pre-mRNA splicing in p53-deficient tumour cells. The E2F family are essential for a wide range of biological processes, including S-phase entry and progression, DNA replication, mitosis, DNA repair, cell differentiation and apoptosis. Perhaps ones of its most important roles in this context, is the p53-dependent down-regulation of the activity of the VEGF promoter under hypoxic conditions. Mendzhanova concludes that their data strongly suggests that E2F1 and SFRS2 are involved in the reduction of tumour neovascularisation.

Whilst there are yet to be any investigations of SFRS2 in endometrial cancer, it is known to be both present in the human endometrium[84, 91] and regulated by sex-steroid hormones[87]. Nie *et al.*. have shown that SFRS2 is present in the primate endometrium (human and rhesus monkey), and have postulated a role in the preparation of a receptive endometrium for implantation[84]. Many genes identified as being vital to implantation require their mRNA to be spliced, which has led to a prediction that SFRS2 has a pivotal role for key modifications required by these proteins at implantation. Progesterone is a key up-regulator of this gene, although no clear mechanism has been established; there does not appear to be a dose-dependent relationship, and the presence of a fetus may be a key regulator[87].

1.3.8 SOX2

SOX2 is another member of the high-mobility group (HMG) box of sex-determining region Y gene family group B[92,93, 94]. SOX2 has a number of vital roles in both the embryonic and adult stem cells, with roles including stem cell determination, pluripotency, differentiation and the maintenance of a cell's ability for proliferative potential[93, 95, 96]. Together with OCT3/4 and NANOG, SOX2 has long been considered to be a master regulator of embryogenesis[96].

The remarkable ability of the human endometrium to regenerate is thought to arise from putative endometrial epithelial stem or progenitor cells located in the basalis glands[97]. These adult stem cells are also implicated in the pathogenesis of the endometrial proliferative conditions including endometriosis and endometrial

adenocarcinoma. Despite their importance, there are no generally accepted markers of the epithelial endometrial progenitor cells, although there are markers such as SSEA-1, CD133 and SOX2 which mark less well differentiated cells[11, 12, 98]. The accumulation of genetic mutations sufficient for malignant transformation may take many years. It has been postulated that the only cells with the necessary life-span, and therefore responsible for carcinogenesis are the adult stem/progenitor cells[99].

There are contradicting reports on the expression of SOX2 in the normal, endometriotic and malignant human endometrium[100, 101]. The expression of a true stem-cell marker would be low in a tissue such as endometrium which only has a small sub-population of less well differentiated cells. As such, the selection of tissue and sensitivity of the detection methods may adversely affect the results.

SOX2 is known to be dysregulated in a variety of cancers, although its role appears to differ in each. It has been reported to be both down- and up-regulated in a diverse range of cancers from those of the gastro-intestinal tract to lung and breast carcinomas. In endometrial carcinoma, studies suggest that hypermethylation of SOX2 and subsequent lower expression is more common in malignant samples than normal controls[96]. This suggests that SOX2 down-regulation may play a role in endometrial carcinogenesis, however, since the overall expression of SOX2 was so low, it seems likely that it only contributes to an overall cascade of events. It must be noted, that other studies have not detected SOX2 in the normal endometrium[101].

In addition to being a stemness-related transcription factor[96], SOX2 is also related to the canonical Wnt/β-catenin pathway[102]. Epithelial-to-mesenchymal transition (EMT) is an essential developmental process whereby previously immobile epithelial cells acquire mesenchymal characteristics[74]. This process also occurs during the migration of cancers from their primary to metastatic sites, as it allows the penetration of both lymphatic and blood vessels. SOX2 has been proposed to regulate EMT via the Wnt/β-catenin pathway, and therefore be a key controller of invasive potential[102]. Li *et al.* propose that SOX2 binds to the promoter region of β-catenin in order to control its transcription[102]. In addition to SOX2 modulating transcriptional responses to Wnt signalling, Wnt signalling can also regulate SOX2 expression[69]. SOX genes are increasingly being recognised as fine-tuners of Wnt signalling via feedback loops[69].

1.3.9 Ki67

Ki67 is a well described marker of proliferation in the endometrial epithelial cells[41], it is known to mark the nuclei of cells in the G1, S, G2 and M phases of the cell cycle. Nuclear β-catenin theoretically marks cells which are actively transcribing downstream Wnt-related genes, whereas Ki67 is a more direct assessment of proliferation.

Chapter 2. Project Aims and Hypotheses

Aim: *To assess the activation of the canonical Wnt/β-catenin pathway via the presence of nuclear β-catenin in normal proliferative phase and post-menopausal endometrium in comparison to endometrial adenocarcinoma.*

Hypothesis: Nuclear β-catenin expression will be seen in occasional cells of the normal endometrium. Most endometrial cancer samples will demonstrate an activated Wnt/β-catenin pathway, therefore increase nuclear β-catenin staining.

Aim: *To assess the expression of nuclear SOX9 in normal proliferative phase and post-menopausal endometrium, in comparison to endometrial adenocarcinoma samples.*

Hypothesis: SOX9 will demonstrate a high-level of staining in the post-menopausal endometrium. A lower level of expression will be seen in the proliferative phase and malignant samples.

Aim: *To assess the expression of the Wnt-related proteins NAP1L1, SFRS2 and SOX2 in normal proliferative phase and post-menopausal endometrium in comparison to endometrial adenocarcinoma.*

Hypothesis: NAP1L1, SFRS2 and SOX2 will be expressed in both the normal and malignant endometrium. Their expression will be related to nuclear β-catenin.

Aim: *To investigate the effects of ovarian steroid hormones on the expression of SOX9 in the Ishikawa endometrial carcinoma cell line.*

Hypothesis: Ovarian steroid hormone treatment will alter SOX9 expression of the Ishikawa cell line.

Chapter 3. Materials and Methods

3.1. Ethics

Ethical approval for the collection of human endometrium was given by the Liverpool Adult Ethics Committee (LREC references; 09/H1005/55 and 11/H1005/4). All patients gave full written informed consent for taking part in the study (Appendix 1).

3.2. Sample groups and collection

All patients were identified by reviewing the elective theatre lists at the Liverpool Women's Hospital. Women who matched the inclusion and exclusion criteria (Table 5 and 6)were approached and asked if they wished to participate in the study by one of the designated personnel in the research team who were trained in Good Clinical Practice (GCP). Since the collected samples were surplus tissue left after surgical specimens had been taken for pathological diagnosis, there were no extra surgical procedures performed than those which were clinically necessary. Informed written consent was obtained together with demographic details including: age, BMI, smoking status, fertility history, use of hormonal treatments, and details of the menstrual cycle.

Table 5: Principal inclusion criteria

Study Group	Inclusion criteria
Proliferative	○ Must be aged 18-50 years. ○ Must be having regular menstrual periods. ○ Must be undergoing surgery for a benign non-endometrial indication at Liverpool Women's Hospital. ○ Must be able to give valid informed written consent.
Post-menopausal	○ Must be over 51 years old. ○ Must be at least 12 months post-menopausal. ○ Must be undergoing surgery for a benign non-endometrial indication at Liverpool Women's Hospital. ○ Must be able to give valid informed written consent.
Cancer	○ Patients must be undergoing hysterectomy for endometrial cancer at the Liverpool Women's Hospital. ○ Patients must be able to give valid informed written consent.

Table 6: Principal exclusion criteria

Study Group	Exclusion criteria
Proliferative	○ Exogenous hormone therapy in the past three months. ○ Pregnancy; current or in the past three months. ○ Breast-feeding; currently or in the past three months. ○ History of endometriosis. ○ History of endometrial pathology. ○ History of infertility.
Post-menopausal	○ Exogenous hormone therapy in the past three months. ○ History of endometriosis ○ History of endometrial pathology
Cancer	○ Previous chemotherapy or radiotherapy.

In benign cases where a hysterectomy was not always being performed and in endometrial cancer samples, a pipelle biopsy was always used. Endometrial biopsies were collected from endometrial cancer patients and some pre-menopausal normal women by trained members of the research team or by a surgeon in theatre under sterile conditions. Once the uterus had been surgically removed in post-menopausal or pre-menopausal women without cancer, it was transferred to a clean trolley. The anterior aspect of the uterus was opened in the coronal plane to expose the uterine cavity. A small, shallow incision was used to remove a full thickness endometrial section containing both the myometrium and endometrium. In women with cancer the uterus was not opened prior to sending to the pathology department due to the possibility of interference with pathological diagnosis.

The pipelle (Laboratoire C.C.D., Paris, France) was introduced into the endometrial cavity, once gentle resistance was felt at the fundal area the inner piston of the pipelle was withdrawn thus creating a vacuum effect which draws the endometrial lining into the pipelle.

The tissue samples for immunohistochemistry were transferred directly into a universal tube containing 15ml neutral buffered formalin (NBF; Sigma-Aldrich, Poole, UK) and incubated for a minimum of 24 hours prior to processing. Formalin fixation preserves the biological sample as close to its natural state as possible. NBF is about 3.7-4% formaldehyde which creates crosslinks between proteins in tissues, resulting in the preservation of tissue as they are found in situ. The samples were

then embedded in paraffin wax using the automated Shandon Citadel 1000 processing machine. It dehydrates then impregnates the formalin fixed tissue with paraffin wax in order to maintain the natural shape and architecture of the sample for long term storage and sectioning. The following processing schedule was used (Table 7).

Table 7: Tissue processing protocol

	Time / mins
10% formalin in neutral buffer	45
60% ethanol	60
70% ethanol	60
90% ethanol	60
100% ethanol	60
100% ethanol	90
100% ethanol	120
Xylene 1	60
Xylene 2	90
Xylene 3	120
Wax 1	150
Wax 2	210

Samples for RNA extraction were immediately transferred to a cryovial containing 500µl of RNA*later* (Applied Biosciences, Warrington, UK), placed on ice, and then stored overnight at 4°C. The RNAlater was subsequently removed and tissue stored at -80°C.

3.2.1 Sectioning and slide preparation

Sectioning

3µm thick sections were cut using a Microm HM355 rotary microtome (Microm Ltds, Thame UK), floated onto a ~40°C waterbath, before being transferred to an aminopropyl triethoxy silane (APES) coated slide and left to dry at room temperature. Slides were stored in covered boxes in order to protect from dust prior to being used.

Preparation of the slide

Slides were placed in a metal staining rack, then either heated at 60°C for one hour or at 37°C overnight. This allowed the removal of moisture from under the section which otherwise can prevent adhesion to the slide. Care was taken to avoid over-baking as this can decrease target staining.

The slides were then deparaffinised using xylene and gradually rehydrated using decreasing percentages of alcohol. Slides are then transferred into tap water. Duration in each of the solutions is outlined below (Table 8).

Table 8: Dehydration protocol

	Time / mins
Xylene 1	10
Xylene 2	10
100% ethanol	5
100% ethanol	5
90% ethanol	1
70% ethanol	1

3.2.2 Immunohistochemistry

Immunohistochemistry (IHC) allows the detection of specific antigens within tissues, the method used here was a two-step technique using the Vector impress reagent kit (Vector, Peterborough, UK) (Figure 8). This system uses a Horseradish Peroxidase (HRP) labelled polymer which has been conjugated with secondary antibodies, which is added following incubation with a primary antibody. Subsequent incubation with 3'3-diaminobenzidine (DAB) + substrate – chromogen leaves a brown-coloured precipitate.

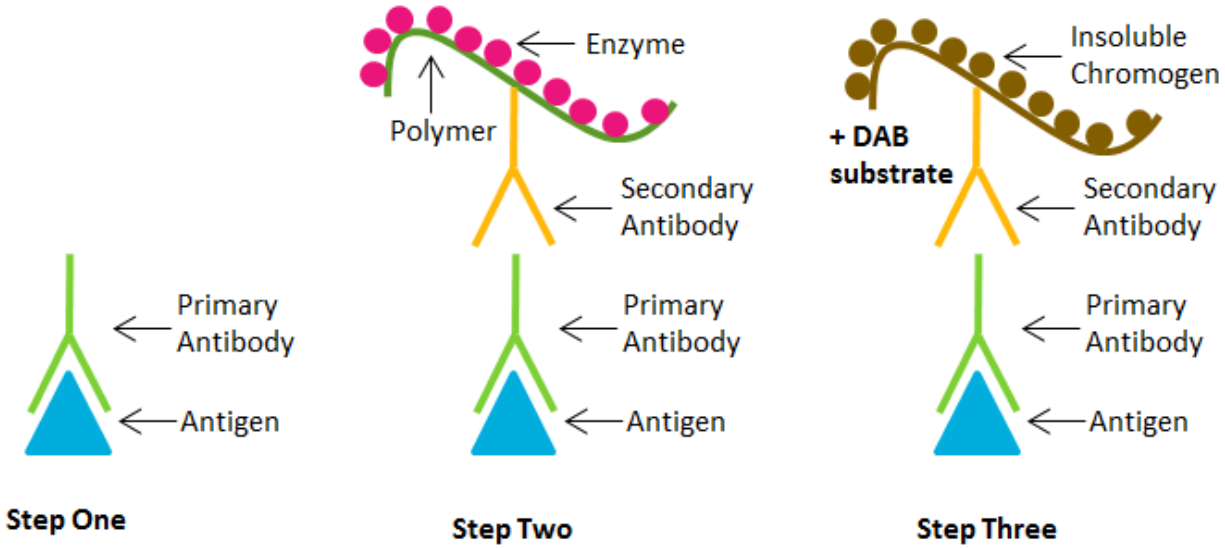


Figure 8: Principles of immunohistochemistry

3.2.3 Antigen retrieval

Formalin-fixation stabilises the tissue proteins by the formation of cross-links; these bonds maintain the tissue morphology, however, they may also alter the antigen's epitopes and/or electrostatic charges. If the epitopes are not restored, then weak or false-negative immunohistochemical staining may occur; restoration of the epitopes allows the epitope to react with the paratope of the antibody.

A 10mM solution of citrate buffer was prepared and adjusted to pH 6.0. Once the buffer was heated to a rolling boil in a stainless steel pressure cooker, the slide racks were immersed, lid engaged and the cooker allowed to come to pressure (indicated by elevation of a button). After a timed period (see Table 10), the pressure cooker was rapidly cooled and the staining racks transferred into tap water.

3.2.4 Immunohistochemistry

Following antigen retrieval, slides were washed in Tris-buffered saline (TBS) for 5 minutes before being immersed in a 0.3% H_20_2 solution in TBS for 10 minutes to quench the endogenous peroxidase activity which could otherwise generate false-positive signals. The slides were then further washed in TBS twice (5 minutes each). A DAKO hydrophobic marker pen (DAKO, Cambridge, UK) was used to encircle the sections on the slides before being placed in a humidified slide chamber. For all antibodies other than mouse monoclonal antibodies, 1-2 drops of 2.5% normal

horse serum block was added to the sections for 20 minutes. Primary antibodies were diluted to the appropriate concentration using antibody diluent (TBS/0.5% bovine serum albumin (BSA)). 50-75µl antibody was added to each section (size dependent) and allowed to incubate in the humidified chamber (Table 10).

Following incubation, the slides were washed twice in TBS (5 minutes each) before 1-2 drops of labelled polymer-HRP were added per section and incubated for 30 minutes at room temperature. The slides underwent two further washes in TBS before a 30µl/1ml DAB chromogen/substrate buffer solution was added to the slides for 10 minutes. The reaction was stopped by immediately immersing the sides in tap water. The slides were counterstained in Gill-2 haematoxylin (Thermoscientific, Loughborough, UK) for 1 minute, washed in tap water then briefly dipped in 1% acid alcohol solution. The slides were then run under cold tap water for 10 minutes before being dehydrated in increasing percentage of alcohol solutions and xylene (Table 9). Cover slips were attached using the Xylene-based Consul Mount (Thermoscientific, Loughborough, UK), and slides allowed to dry. Care was taken at all stages to prevent the sections from drying out which may result in non-specific staining.

Table 9: Dewaxing protocol

	Time / mins
70% ethanol	1
90% ethanol	1
100% ethanol	3
100% ethanol	3
Xylene 1	5
Xylene 2	10

Table 10: Immunohistochemistry antibody conditions

	Antibodies					
	SOX9	**β-catenin**	**NAP1L1**	**SFRS2**	**SOX2**	**Ki67**
Antigen retrieval / min	4	2	1	1	1	4
Host species	Goat	Rabbit	Rabbit	Rabbit	Mouse	Mouse
Horse serum	Yes	Yes	Yes	Yes	No	No
Concentration	1:200	1:400	1:4000	1:500	1:25	1:200
Incubation time	Overnight	2 hours	Overnight	Overnight	Overnight	Overnight
Incubation temperature	4°C	25°C	4°C	4°C	4°C	4°C
Supplier Details	R&D Systems, Abingdon, UK	Cell Signalling Technology, Herts, UK	Abcam, Cambridge, UK	Abcam, Cambridge, UK	R&D Systems, Abingdon, UK	Vision Biosystems, Novocastra, Newcastle, UK
Supplier code	AF3075	9582S6	Ab33076	Ab28478	MAB2018	NCL-Ki67
Clone					245610	MM1

3.2.5 Controls

To ensure that staining intensity remained constant between staining runs an internal control was used (Table 11). Consecutive sections of positively staining sample were cut and included in each run. The stained slides were then compared to ensure consistency of results. A positive control was used to ensure appropriate positive staining intensity was observed, and a negative control (IgG matching the primary antibody) was performed on at least one of the sections being stained in each run to ensure the staining was specific.

Table 11: Controls for immunohistochemistry

Antibody	External positive control	Internal Positive control	Negative control
B-catenin	Liver	EndoCa 15-1	Rabbit IgG
SOX9	Prostate	EndoCa 15-1	Goat IgG
SFRS2	Gut	EndoCa 23-2	Rabbit IgG
NAP1L1	Gut	EndoCa 23-2	Rabbit IgG
SOX2	Endometrial cancer	EndoCa 14-5	Mouse IgG
Ki67	Tonsil	EndoCa 15-1	Mouse IgG

3.2.6 Haematoxylin and eosin staining for dating and staging

All specimens were stained using haematoxylin and eosin in order to analyse the histopathology of the sections. The haematoxylin stains the cell nuclei blue/black, whereas the eosin stains the cell cytoplasm and connective tissue fibres shades of pink; this demonstrates the tissue structures. The sections were reviewed blinded by a Consultant Gynaecologist and/or a pathologist within the department, and correlated with the pathology report in order to establish histological dating. Any discrepancies were reviewed. This was used in conjunction with the information from patients, removing any recall bias as some patients struggle to remember their last menstrual period. In the case of the endometrial cancer samples, this ensured that the sample being stained contained malignant tissue. Endometrial carcinomas are typically heterogeneous and therefore it is important to know the grade of the specific section being analysed.

3.2.7 Image analysis

In order to be able to assess whether the Wnt-related proteins were differentially expressed in the study groups, both the staining intensity and patterns needed to be quantified. For some of the proteins such as β-catenin both the intra-cellular location and staining intensity were required to be assessed, whereas for other such as SFRS2 only the intensity was necessary. For this reason, several different analysis methods were employed.

3.2.8 Image capture

The stained sections were visualised and captured using Nikon Biophot Micoscope and camera head (Nikon, Tokyo, Japan). For post-menopausal and proliferative samples, the functional layer of the endometrium was located and 10 consecutive high-power views were captured. Where full-thickness sections were used, care was taken to ensure that only glands within the functionalis were captured. This was to ensure that the control groups were as much as direct a comparison as possible for the pipelle samples. For malignant samples, 10 consecutive high-power views containing malignant cells were captured.

3.2.8.1. Percentage of positive cells

For the nuclear proteins Ki67 and SOX9, and proteins such as β-catenin and SOX2 where the intra-cellular location was important, the percentage of positive cells was assessed. Initial quantification was performed using the ImageJ plug-in cell counter (www.rsbweb.nih.gov/ij/ version 1.48a) (Figure 9).

Firstly, the endometrial glands were isolated using the selection tool. Then, cells were individually selected in either red (positive) or blue (negative) and the totals collected. This gives the percentage of positively stained cells within the glands. Extra care was taken in the malignant samples to ensure that only malignant cells were assessed.

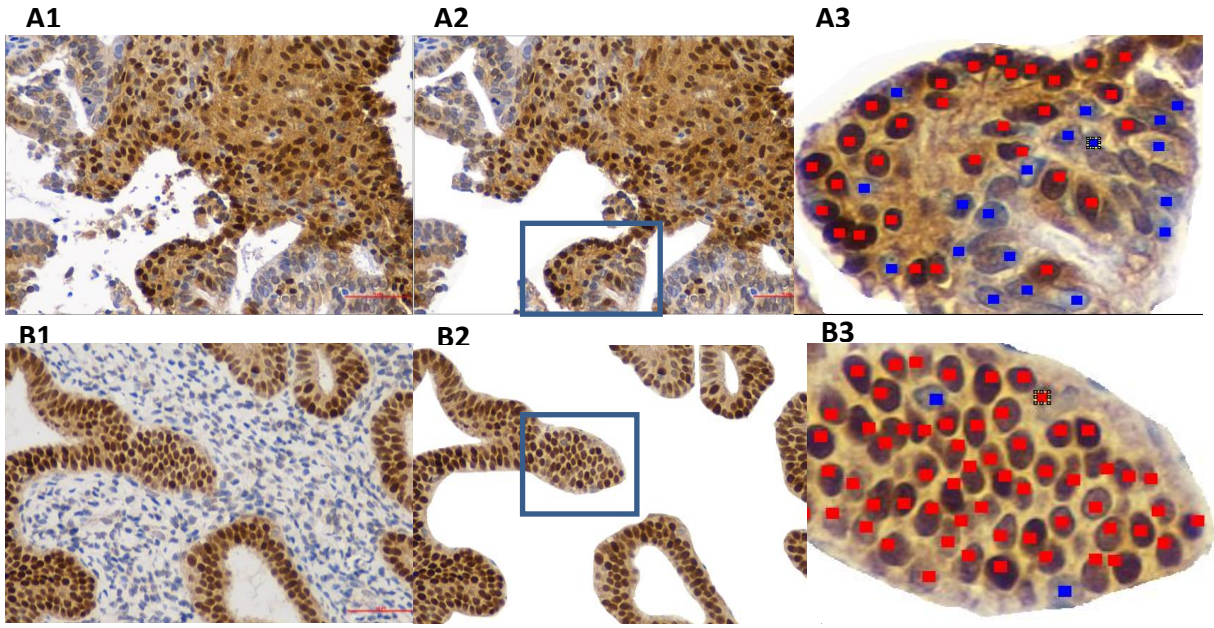


Figure 9: Cell counting using ImageJ. 1) Original image for analysis. 2) Removal of stroma. 3) Nuclei marked as either positive (red) or negative (blue) in A) β-catenin staining in a cancer sample. B) SOX9 staining in a post-menopausal sample

This technique was utilised for β-catenin and SOX2. Following scoring all of the sections for these proteins, it was possible to accurately estimate the percentage of positively stained cells. Where percentages of positive cells have been estimated (SOX9 and Ki67), they are estimated to within a 10 percentile band. An example of validating this technique is shown below (Table 12). Where discrepancies were noted (highlighted in green), they were acceptably small.

Table 12: Comparison of calculated to estimated percentage positive cells

Image	Estimated percentage positive cells	Calculated percentage positive cells
1	<10%	9.9
2	50-60%	51.3
3	40-50%	44.0
4	70-80%	77.3
5	90-100%	89.7
6	30-40%	36.6
7	<10%	0.91
8	90-100%	95.5
9	<10%	5.62
10	30-40%	27.9
11	60-70%	64.8
12	<10%	4.6
13	40-50%	47.6
14	70-80%	71.8
15	30-40%	41.3

3.2.8.2. Image J colour deconvolution and area measurement

The surrounding stroma was discarded using the method outlined above. ImageJ has built-in vectors for haematoxylin and DAB which separate the blue (haematoxylin) from the brown (DAB). Auto-threshold colour deconvolution tool was applied, and the area measured using the 'area measurement' function (Figure 10).

$$\text{Estimate of stained area} = \frac{\text{Area of brown in 10 high-powered fields}}{\text{Area of blue in 10 high-powered fields}}$$

This is a validated and published method for accurately estimating positive staining[103]. This method does not distinguish nuclear from cytoplasmic staining, but as it combines both, it provides an objective score for both NAP1L1 and SFRS2.

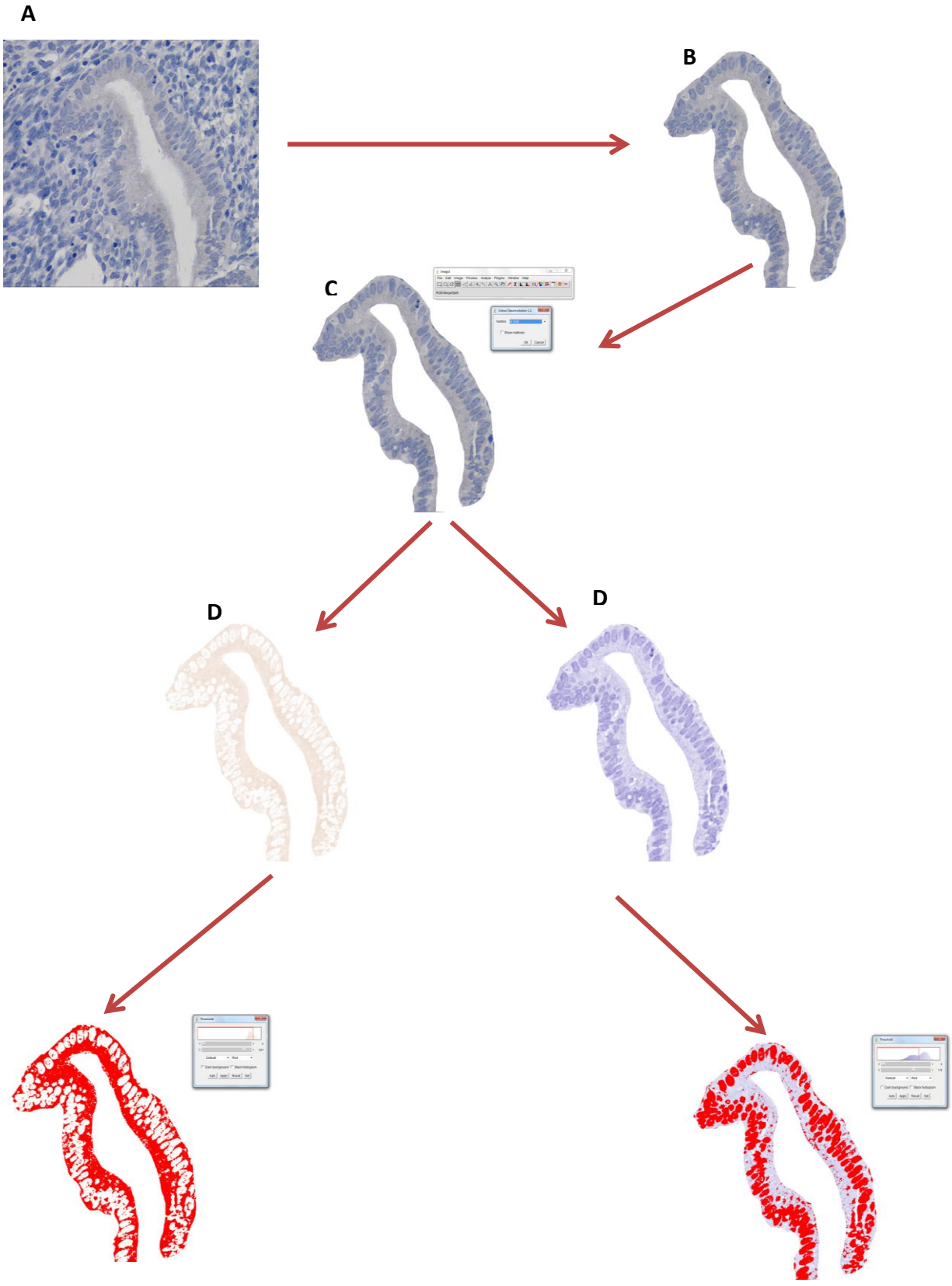


Figure 12: Colour deconvolution and area measurement using Image J. A) Original image for analysis. B) Stroma removed. C) Selecting the haematoxylin and DAB (HDAB) vectors. D) Brown/DAB (left) and blue/haematoxylin (right) staining. E) Thresholding of area to be measured.

3.2.8.3. Modified QuickScore

The modified QuickScore technique combines the intensity and proportion of staining to provide a semi-quantitative overall score for the stain (Table 13). The entire functional layer or malignant area of cancer samples was assessed at high power and a score assigned.

Table 13: QuickScore criteria

Intensity		**Percentage of positive cells (%)**	
0	Absent	1	<25%
1	Weak	2	25-50%
2	Moderate	3	50-75%
3	Strong	4	>75%

Results are scored by multiplying the percentage of positive cells by the intensity, the maximum score is 12. For example if 25% of a sample was negative, 50% was weakly stained and 25% moderately stained, the score would be:

(Absent x <25%) + (Weak x 50%) + (Moderate x <25%) = QuickScore

(0 x 1) + (1 x 2) + (2 x 3) = 8

Prior to scoring, all samples were reviewed in order to establish what staining intensity would constitute weak, moderate and strong staining. Images were taken as described previously, and used as guide.

3.3. Cell culture

The Ishikawa cell-line was derived from a well-differentiated grade 1 human endometrial adenocarcinoma (obtained from Public Health England (Salisbury, UK)) and was used to assess the functional role of SOX9 in vitro.

The cells were maintained in DMEM/F12 (Sigma, UK), 10%FBS (BioSera, UK) supplemented with L-glutamine (2mM final, Sigma) and the antibiotic, Primocin^{TM} (1/500 in media; InvivoGen).

For experiments involving steroids the cells were maintained in DMEM/F12 (phenol-free, Life Technologies, UK) with 5% charcoal-stripped FCS (Sigma) for at least 72 hours prior to the experiment. Cells were seeded at 2x 10^5 cells/well in 6-well tissue culture dishes (Nunc, Fisher Scientific, UK) in media with or without steroid hormones or growth hormones individually or in combination. All experiments were performed in triplicate.

Five ovarian steroid hormone treatments were employed in order to assess their effect on SOX9 expression (Figure 11). *Estradiol* (E_2), the highly potent synthetic progestagen medroxyprogesterone acetate (MPA), a combination of both E_2 and MPA, fibroblast growth factor (FGF) and the androgen (5α-dihydrotestosterone). Although not an ovarian hormone, FGFs play a key role in modulating the effects of progesterone on the endometrium, as well as modulating SOX9 in other tissue systems[68].

The hormones were made as 1mg/ml stocks in ethanol or methanol and used at the indicated concentrations for 72 hours: 17 beta-estradiol (E2; 10^{-8}M; Sigma), medroxyprogesterone acetate (MPA; 10^{-6}M; Selleckchem through Stratech Scientific, UK), 5 alpha-dihydrotestosterone (DHT; 10^{-6} M; Sigma). Cells were also cultured in fibroblast growth factor (FGF, basic 146 aa; R&D Systems) at 50ng/ml. For RNA isolation, cells were harvested into TRIzol (Life Technologies, UK) 1 ml/ well and stored at -80^{o}C prior to RNA extraction.

Tissue culture experiments were performed in conjunction with Anthony Valentijn.

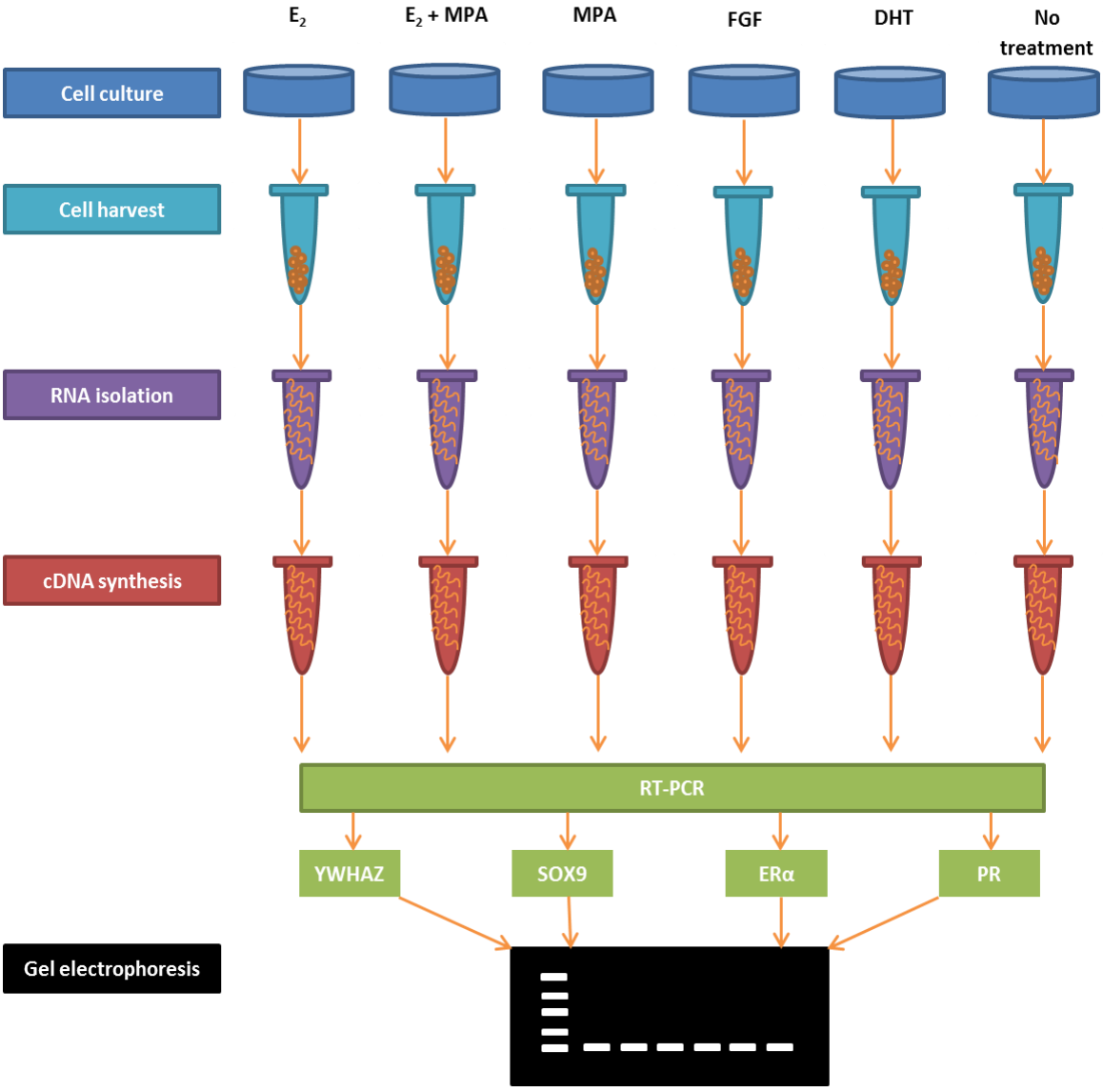


Figure 13: Flow diagram of the cell culture experiments to assess the effect of hormonal treatments on the transcription of SOX9, ERα and PR.

3.4. RNA isolation

In order to extract RNA the Pure Link RNA mini kit (Ambion, Life Technologies, Paisley, UK) was used. TRIzol is a monophasic solution which contains phenol, guanidine isothiocynate and other components which disrupt cells and dissolves cellular components whilst promoting inhibition of RNases and maintaining RNA integrity. Where fresh tissue was used, the tissue was first homogenised using Ultra-turrax (IKA Labortechnik, Staufen, Germany) for 2 mins, in 1ml TRIzol per 50-100mg tissue to ensure that the TRIzol can work most effectively. The adherent cells from culture were incubated in 1 ml TRIzol per 10 cm^2 for 5 minutes and disrupted using a pipette tip. Following these initial steps, the RNA extraction was performed using the same method.

The cell lysates were incubated with TRIzol at room temperature for 5 minutes to allow complete disassociation of nucleoprotein complexes. 0.2mL chloroform per 1 mL TRIzol was added to each tube and shaken vigorously for 15 seconds prior to being centrifuged at 12,000 x g for 15 minutes at 4°C. This separated the mixture into a lower red phenol-chloroform phase, an interphase and an upper aqueous phase which contained the RNA. This upper phase was transferred to a fresh RNase-free tube, to which an equal volume of 70% ethanol was added and vortexed.

The PureLink RNA mini kit utilises a silica-based membrane in a spin-column, to which the RNA binds during purification. The membrane is then washed using proprietary buffers included in the kit and eluted using RNase-free water. Up to

700µl of sample was transferred to a spin cartridge, spun at 12, 000 x g for 15 seconds at room temperature and the flow-through discarded. This was repeated until the entire sample has been processed. 700µl of Wash Buffer I was added to the spin cartridge, spun under the same conditions and the collection tube replaced. Next 500µl of Wash Buffer II was added to the spin column, centrifuged for 15 seconds at 12, 000 x g for 15 seconds and the flow-through discarded. This was repeated once. The membrane was dried by centrifugation for 1 minute at room temperature at 12, 000 x g. The collection tube was replaced with a recovery tube and 30µl RNase-free water was placed in the centre of the spin cartridge before being centrifuged at 12, 000 x g at room temperature for 2 minutes. The recovery tube then contained the purified total RNA which should be stored at -80°C unless synthesized immediately.

3.5. cDNA synthesis

The reaction to convert messenger RNA (mRNA) template into complementary DNA (cDNA) is catalysed by reverse transcriptase. The RNA was treated with DNase to ensure the removal of all genomic DNA. The reverse transcriptase enzyme operates on a single strand of mRNA, to which oligo dTs bind acting as a primer for the reverse transcriptase to bind. The addition of DNA bases allows the generation of complementary DNA based on the pairing of RNA base pairs (A, U, G and C) to their DNA complements (T, A, C and G respectively).

cDNA was synthesized from the extracted RNA using the Avian myeloblastosis virus (AMV) First Strand cDNA Synthesis Kit (New England BioLabs Inc, Hitchin, Herts, UK) was used. 1µl RNA, 2µl $d(T)_{23}VN$ (50µM) and 5µl nuclease-free water were added to sterile RNase-free microfuge tubes, denatured for 5 minutes at 70°C. The tubes are briefly microfuged and placed on ice to improve the cDNA yield for long messenger RNAs. 10µl of AMV reaction mix and 2µl AMV enzyme mix (or water for no enzyme control) is added to each tube. The cDNA synthesis reaction is then incubated at 42°C for one hour before the enzyme is inactivated at 80°C for 5 minutes. 30µl water is added to each tube to dilute the reaction to 50µl. The cDNA was stored at -20°C in the short term; -80°C for long-term storage.

The cDNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher) (Table 14). The 260/280nm absorbance ratio was also given which reflects

the RNA purity. The samples were diluted to an approximate concentration of 100ng/µl.

Table 14: Quantification of RNA and cDNA concentration and quality

Sample	RNA ng/µl	RNA A260/A280	cDNA ng/µl	cDNA A260/A280
E_2 1	1274.7	2.09	621.6	1.80
E_2 2	1273.5	2.07	836.7	1.84
E_2 3	752.9	2.04	601.3	1.80
E_2 + MPA 1	1055.5	2.06	620.7	1.80
E_2 + MPA 2	1164.2	2.09	563.5	1.81
E_2 + MPA 3	1074.6	2.07	1032.7	1.84
MPA 1	1262.2	2.08	574.4	1.81
MPA 2	1416.8	2.12	869.3	1.83
MPA 3	1254.6	2.08	1140.0	1.86
FGF 1	908.9	2.01	898.4	1.83
FGF 2	657.9	2.01	976.6	1.87
FGF 3	1276.6	2.02	598.0	1.81
DHT 1	756.2	2.02	807.2	1.83
DHT 2	920.0	2.03	659.4	1.80
DHT 3	722.0	2.02	645.5	1.84
Vehicle 1	638.4	1.99	736.4	1.84
Vehicle 2	817.7	2.05	583.1	1.79
Vehicle 3	640.8	1.98	623.6	1.80
Vehicle 4	1051.7	1.97	786.1	1.82
Vehicle 5	466.1	1.98	624.2	1.80
SPCEX123	1447.1	1.87	728.3	1.82
ESC			1662.0	2.19
Prostate			716.3	1.79

3.6. RT-PCR

Polymerase chain reaction (PCR) is a technique which amplifies and reproduces defined sections of precise genetic material due to thermal cycling.

There are three steps to the PCR reaction:

1. **Denaturing:** cDNA is heated to 95°C to break the hydrogen bonds which unite the complementary strands.

2. **Annealing:** Primers with complementary sequences to those at either end of the region of DNA to be amplified attach (anneal) to their complementary sequences. Too high a temperature could prevent primer binding, and if too low the binding would be non-specific. 56°C was used in this project.

3. **Extension:** the temperature is lowered to 68°C (as specified by the HotStart Taq mix) to allow the Taq DNA polymerase enzyme to work optimally. The enzyme attaches to each primer and incorporates complementary nucleotides into the single DNA strand. This creates a copy of the region specified by the primers.

These steps were repeated 30-35 times in this experiment. With each cycle, each single strand of double stranded DNA template was amplified into two separate double-stranded DNA. Once these were separated during denaturing, they became

available as a template for the next amplification. This results in exponential amplification of the original DNA template (Figure 12).

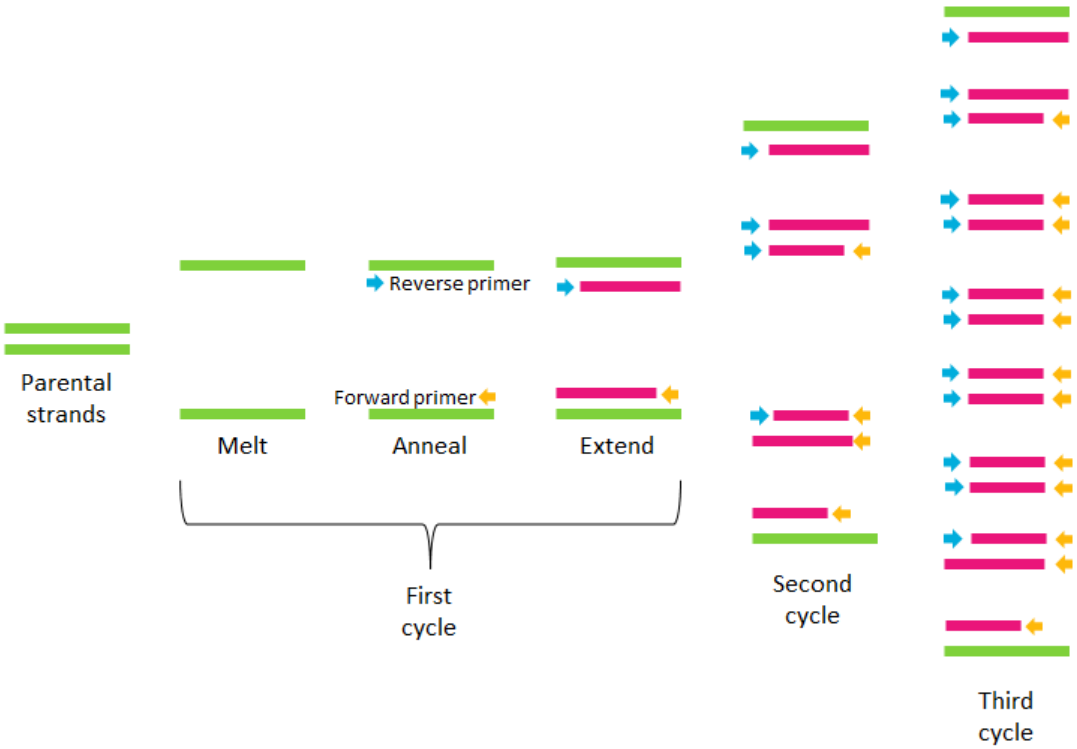


Figure 12: The principles of RT-PCR

3.6.1 RT-PCR

A primer mix containing both forward and reverse primers was prepared at a concentration of 6.25 µM for each primer pair.

12.5µl	HotStart Taq 2x master mix (New England Biolabs Inc, Hitchin, Herts, UK)
1µl	Primer mix
10.5µl	Nuclease free water

A master mix stock solution was prepared by adding x times amounts listed above and 24µl from this pipetted into UV treated PCR tubes. 1µl of cDNA was added to each tube. Attention was taken to prevent contamination of solution and tubes.

The following programme was used for the RT-PCR reaction:

95°C	30 seconds	
95°C	20 seconds	30-35 cycles
56°C	20 seconds	
68°C	30 seconds	
68°C	5 minutes	
4°C	Hold	

Primer sequences and product sizes are outlined below (Table 15).

Table 15: RT-Primers

Gene Name	Sequences		Size	TM° C	PCR product size (base pair (bp))
YWHAZ	Fwd	5'-CGTTACTTCGCTGAGGTTGCC-3'	21	67.8	69
	Rev	5'-GTATGCTTGTTGTGACTGATCGAC-3'	24	64.6	
SOX9	Fwd	5'-GTACCCGCACTTGCACAAC-3'	19	64.3	74
	Rev	5'-TCTCGCTCTCGTTCAGAAGTC-3'	21	63.8	
SOX2	Fwd	5'-CGAGATAAACATGGCAATCAAAAT-3'	24	64.6	85
	Rev	5'-AATTCAGCAAGAAGCCTCTCCTT-3'	23	65.3	
Human ERα	Fwd	5'-TGATTGGTCTCGTCTGGCG-3'	19	67.6	101
	Rev	5'-CATGCCCTCTACACATTTTCCC-3'	22	66.3	
Human PR	Fwd	5'-CAGTGGGCGTTCCAAATGA-3'	19	67.1	83
	Rev	5'-TGGTGGAATCAACTGTATGTCTTGA-3'	25	66.3	

The housekeeping gene YWHAZ was chosen as it has been demonstrated to be a stable in the endometrium when analysed using RT-PCR[104]. It is therefore a suitable and useful housekeeping gene for this experiment.

In order to ensure that the PCR reaction had worked, the following positive controls were used (Table 16).

Table 16: Positive controls for RT-PCR

Gene Name	Positive control	
YWHAZ	SPCEX123	Primary endometrial sample
SOX9	PC3	Prostate cancer cell line
SOX2	H7	Embryonic stem cell line
Human ERα	SPCEX123	Primary pre-menopausal endometrial sample
Human PR	SPCEX123	Primary pre-menopausal endometrial sample

3.6.2 Agarose gel

In order to visualise the PCR products, electrophoresis was performed using a 1% agarose gel with SYBR Safe. 4g of agarose powder (Sigma Aldrich, Poole, UK) and 40µl SYBR Safe (Invitrogen Ltd., Paisley, United Kingdom) were added to 400ml 1x Modified Tris-Acetate EDTA (TAE) buffer (Millipore Corporation, Nottingham, UK) and heated in a microwave until completely dissolved. The solution was then poured carefully into a gel tray and air bubbles removed using a pipette tip before being left to set. To prepare the PCR products for electrophoresis, 5µl of loading buffer (Type II loading buffer, Abgene, Epsom, UK) was added to each tube and mixed well. When fully cooled, sufficient electrophoresis buffer (1x TAE) was poured over the gel to cover both the gel and electrode attachments and the combs removed. 5µl of the PCR product solution was added to each well using fresh pipette tips for each pipette sample to prevent cross-contamination, 1µl of a 50 bp

DNA molecular weight marker (50bp ladder, New England Biosciences) was placed in the first well.

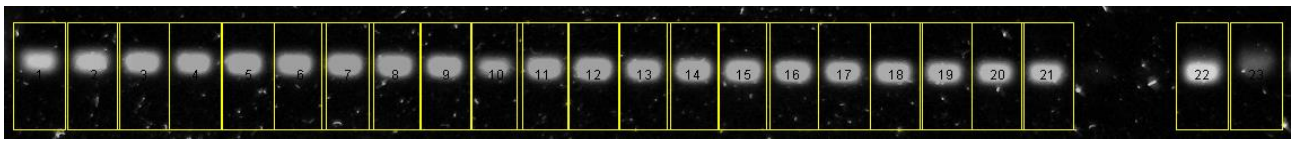
Once the gel had been loaded, the electrodes were connected to the supply unit and run at 120 volts for 30-45 minutes, or until the DNA ladder was fully separated. The gel was then visualised using the ChemiDoc-It ^{TS2} Imager (UVP systems, Cambridge, UK) and photographs taken. This allows visualisation of the PCR products to assess whether there has been any contamination in the negative controls, that the products correspond to the correct size, and to quantify the products.

3.6.3 Analysis of PCR

Following gel electrophoresis, the PCR bands were analysed using ImageJ software (Figure 13). Each band was isolated, the pixel intensity calculated, and a histogram created (x axis = pixel location, y axis = intensity value) using the gel analysis function. The area under the curve represents the amount of signal in the band. This was repeated three times for each band and the average calculated.

In order to compensate for intra- and inter-kinetic RT-PCR variations (sample-to-sample and run-to-run variations) all target gene expression levels were normalised to the housekeeping gene YWHAZ. For each sample, the signal for each test gene was divided by the YWHAZ signal for the corresponding sample. This value was used for statistical analysis.

A



B

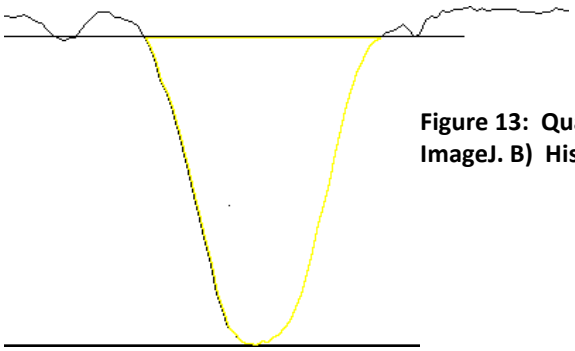


Figure 13: Quantification of RT-PCR. A) Individual bands isolated using ImageJ. B) Histogram of PCR band with area below the curve highlighted.

3.7. Statistical analysis

Data was analysed using SPSS statistics software (Version 20, IBM) and GraphPad Prism software (version 6). Immunohistochemistry results were compared to the normal control (post-menopausal and proliferative) groups. Global differences were assessed using the non-parametric Kruskal-Wallis (KW) test, if this suggested that there were differences between the groups, they were investigated using the non-parametric Mann Whitney (MW) test. Correlations between protein expression in the primary samples was assessed using the Spearman Rank Correlation statistical test (SR). PCR results were analysed in a similar way using the untreated (vehicle) cells as the control. Statistical significance was set at $p < 0.05$. Advice on appropriate statistical tests was gained from Dr Anna Hart (University of Lancaster).

Chapter 4. Results

4.1. Patient demographics

This study was performed over a 12 month period from September 2012 – August 2013. 31 cancer, 10 normal post-menopausal (PM) and 10 normal proliferative phase (PP) samples were collected according to the criteria outlined previously and analysed using immunohistochemistry (Table 17).

Table 17: Patient demographics

Study Group	Sample number		Age	BMI	Parity	Disease stage
Grade 1	10	*Mean (±SD)* *Median* *Range*	63 ± 3 63 53 - 83	38.7 ± 10.5 39.6 23.7 – 53.2	3 ± 2.1 3 0 - 5	1 ± 0.3 1 1 - 2
Grade 2	13	*Mean (±SD)* *Median* *Range*	64 ± 10 60 51 - 79	29.5 ± 5.0 28.9 21.7 – 37.9	3 ± 1.7 3 0 - 6	1 ± 0.9 1 1 - 3
Grade 3	8	*Mean (±SD)* *Median* *Range*	71 ± 7 72 60 - 80	29.4 ± 6.4 27.7 23.9 – 42.7	2 ± 2.0 2 0 - 6	2 ± 1 1 1 - 3
Post-menopausal	10	*Mean (±SD)* *Median* *Range*	72 ± 6 75 62-78	28.2 ± 3.4 27.5 23.7 – 35.8	2 ± 0.7 2 2 - 4	-
Proliferative	10	*Mean (±SD)* *Median* *Range*	39 ± 5 39 30-42	27.2 ± 7.5 27.0 17.5-45.5	3 ± 1.5 3 0 - 5	-

The available demographic details of the patients were analysed using Kruskal-Wallis test and no significant difference was found between any of the study groups for BMI (KW p=0.131) or parity (KW p=0.595). All women with cancer were post-menopausal, thus as expected, the age of the proliferative group was significantly lower than all grades of cancer (KW p = <0.0001) and post-menopausal (KW p = <0.0001). However there was no difference between the grades of cancer and post-menopausal groups.

Cancer samples were graded by the pathology department at the Royal Liverpool University Hospital (RLUH) and internally verified by a second independent pathologist within the department of Women's Health. Details of staging were obtained from the clinical notes (Figure 18). No difference was observed between the grades of cancer for FIGO stage.

Table 18: FIGO stage of all cancers

FIGO Stage	Sample Number	Percentage (%)
IA	16	57
IB	5	18
II	2	7
IIIA	3	11
IIIB	1	4
IIIC1	1	4
IIIC2	0	0
IVA	0	0
IVB	0	0

No grade IIIC2 or grade IVB cancers were available for practical reasons; patients with metastatic disease are rarely operated on at LWH without prior treatment with either radio- or chemotherapy.

4.2. Results: Immunohistochemistry

51 primary endometrial samples (31 endometrial adenocarcinomas, 10 normal proliferative phase and 10 normal post-menopausal) were assessed for the expression of 6 proteins (SOX9, Ki67, β-catenin, SOX2, SFRS2 and NAP1L1) using immunohistochemistry as previously outlined. Differential expression between the malignant samples and the normal controls was evaluated using the Kruskal-Wallis statistical test. Differences between individual groups were appraised using the Mann-Whitney statistical test as previously described. Correlations between protein expression were assessed using the Spearman Rank Correlation test. Results were determined to be statistically significant where $p<0.05$.

4.2.1 β-catenin

Wnt/β-catenin signalling is important in the endometrium, maintaining the balance between proliferation and differentiation. Enhanced Wnt/β-catenin signalling is implicated in endometrial carcinogenesis. The transcription factor β-catenin enables the transcription of the downstream proliferative genes of the canonical Wnt pathway, when it is located in the nucleus and can therefore be used as a surrogate for determining Wnt activation[105].

There are three possible staining patterns for β-catenin, which reflects its different functions; the cell nucleus (A), cytoplasm (B), or adherens cell junction (C) (Figure 14). For the purposes of assessing the canonical Wnt/β-catenin pathway, only nuclear and cytoplasmic staining was assessed.

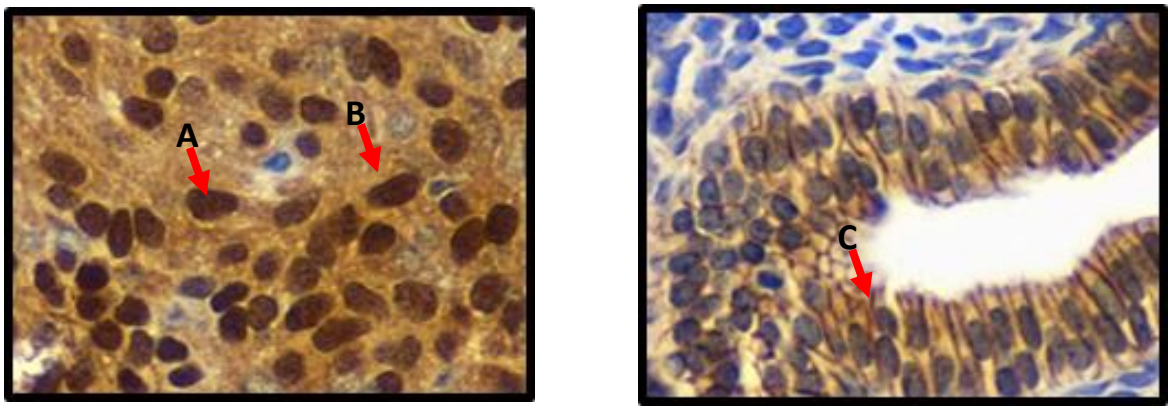


Figure 14: Staining patterns of β-catenin. A) Nuclear B) Cytoplasmic C) Junctional

β-catenin can be seen extensively in both the cytoplasm and nucleus of epithelial cells, and therefore two scoring systems were used which quantified these separately. β-catenin was not observed in the stromal compartment. Overall, nuclear staining was differentially expressed between the groups (KW p=0.049); grade 1 cancers showed significantly more nuclear staining than PP samples (MW p=0.023) (Figure 15). However, although statistically insignificant at the 95% confidence interval, there is a clear trend that nuclear β-catenin is up-regulated in cancers in comparison to the non-malignant controls. 13/31 malignant samples expressed nuclear β-catenin in comparison to 2/20 benign controls. Furthermore, nuclear β-catenin expression rises with increasing grade of cancer.

Cytoplasmic staining was also differentially expressed across the groups (KW p=0.033). Grade 2 cancers demonstrated lower staining levels than both PP (MW p = 0.049) and PM (MW p=0.015) samples. There would appear to be a similar, but opposing trend to nuclear staining, whereby cytoplasmic staining decreases with increasing grade. However, this is far less obvious, and no conclusion could be drawn from this.

The relationships between β-catenin expression (nuclear and cytoplasmic) and Wnt-related proteins were investigated using the Spearman's Rank Correlation test.

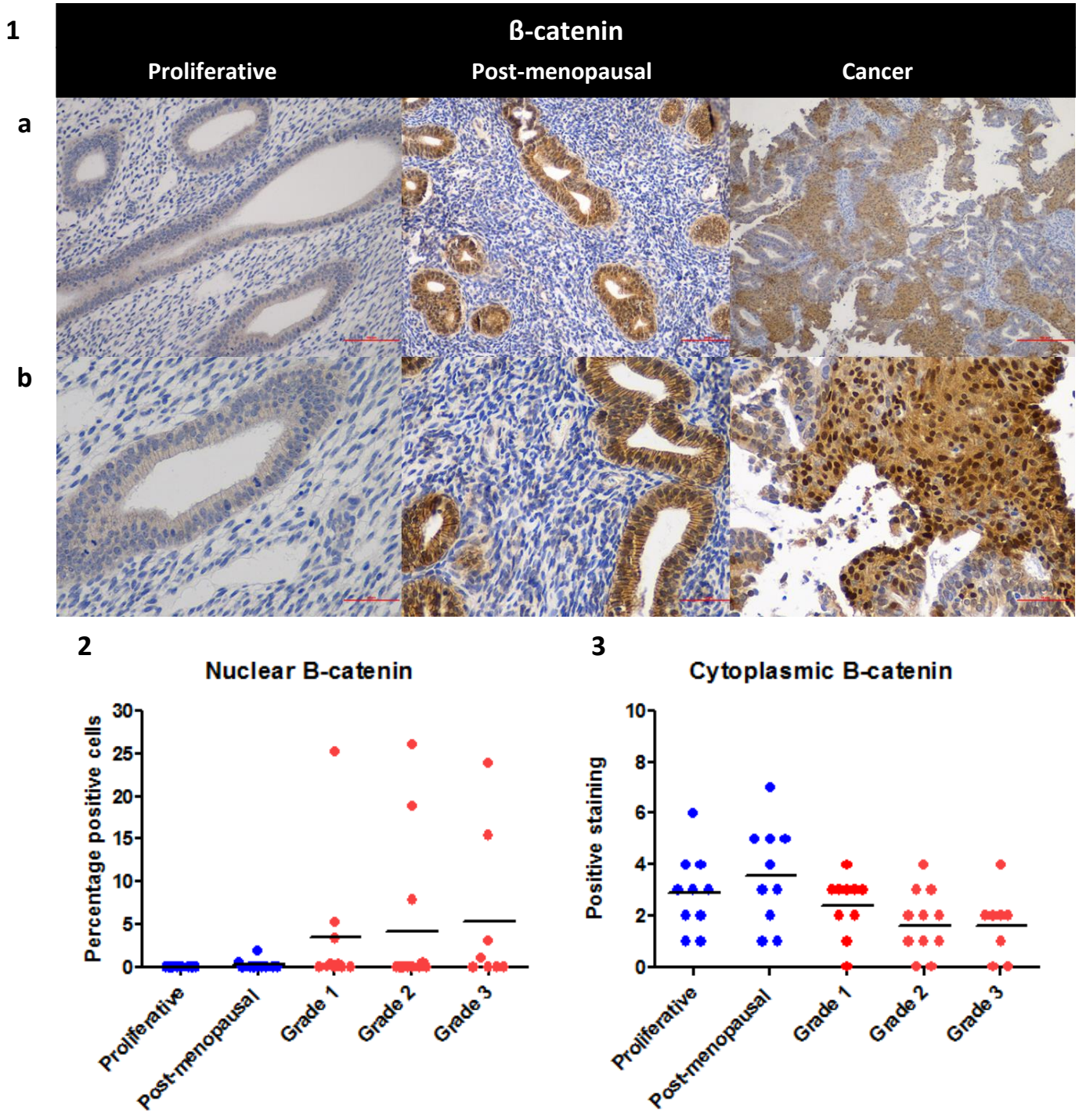


Figure 17: Immunohistochemical staining for β-catenin. 1) Immunohistochemical staining for β-catenin of normal proliferative phase endometrium (n=10), post-menopausal endometrium (n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification. B) 400x magnification. 2) Scatter plots showing median, inter-quartile range and range for percentage positively stained nuclei for nuclear β-catenin. 3) Scatter plots showing median, inter-quartile range and range for QuickScore of cytoplasmic β-catenin staining.

4.2.2 Ki67

Ki67 is seen in the nucleus of proliferating cells in both the stroma and glands of the endometrium. Only staining in the endometrial glands was assessed as this is the location of origin of endometrial cancer. High-grade endometrial cancers also lose their stroma, and therefore discounting the stroma of normal tissues provides the most appropriate comparison for malignant tissue. Ki67 staining was up-regulated compared to PM controls in all cancer grades (MW $p<0.0001$) (Figure 16). PP samples also showed increased Ki67 staining compared to PM samples (MW $p=0.005$). The endometrium becomes atrophic after menopause due to ovarian failure; a weak or non-existent proliferative pattern is therefore to be expected. With the development of adenocarcinoma, the endometrium regains its proliferative potential, therefore increased Ki67 staining. No statistical difference was observed between cancers and PP samples, or between the grades of cancers.

The PP samples demonstrated a wide range of Ki67 staining, with some areas having almost every cell positively stained, and other areas with less than 1% staining. On viewing the PP sections at low power, Ki67 was more abundant in the functionalis compared to the basalis, although this was not objectively quantified. One explanation for the varying Ki67 density could be whether they are early or late proliferative phase; this was not possible to assess.

Nuclear β-catenin is associated with transcription of genes associated with proliferation. A higher proliferative index would be expected in regions containing

nuclear β-catenin than those without. Surprisingly, there was no correlation between nuclear β-catenin and the proliferation marker Ki67 (data not shown).

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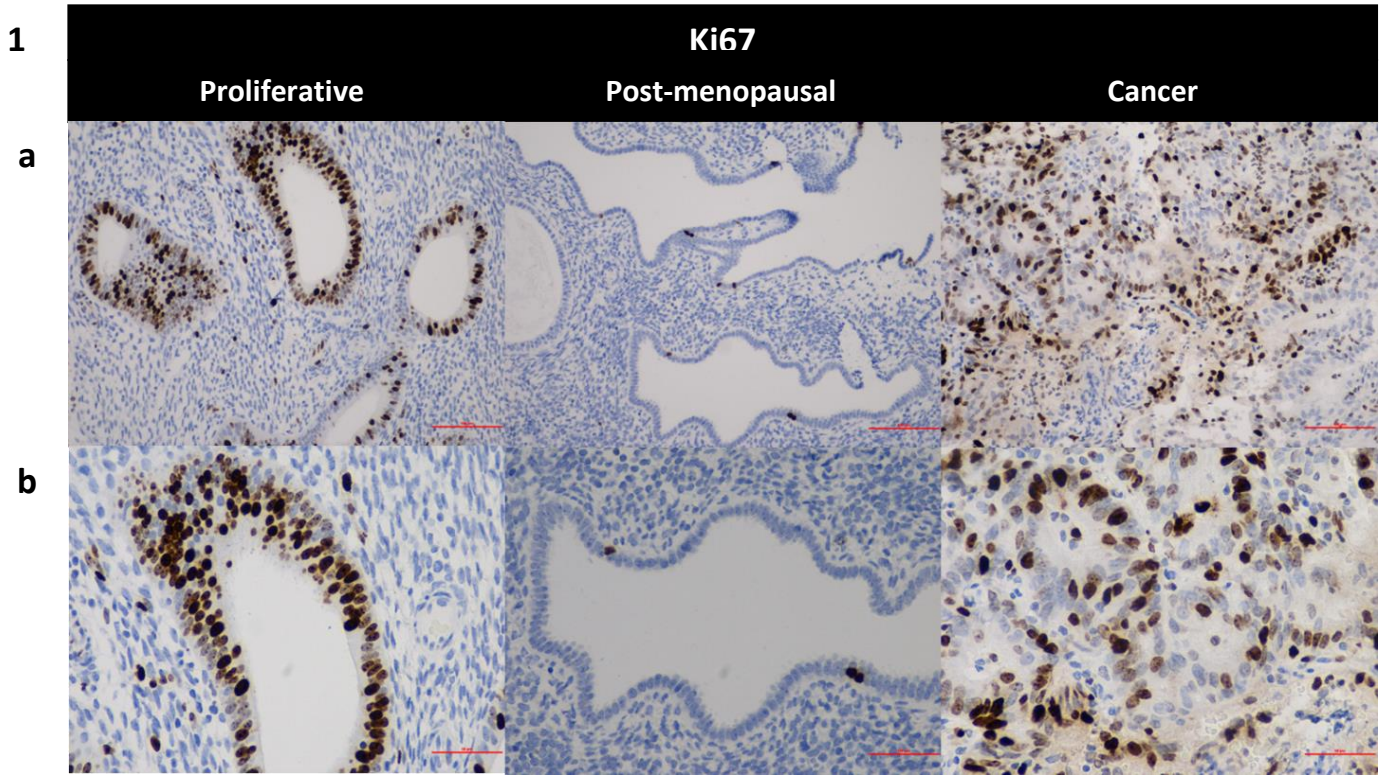

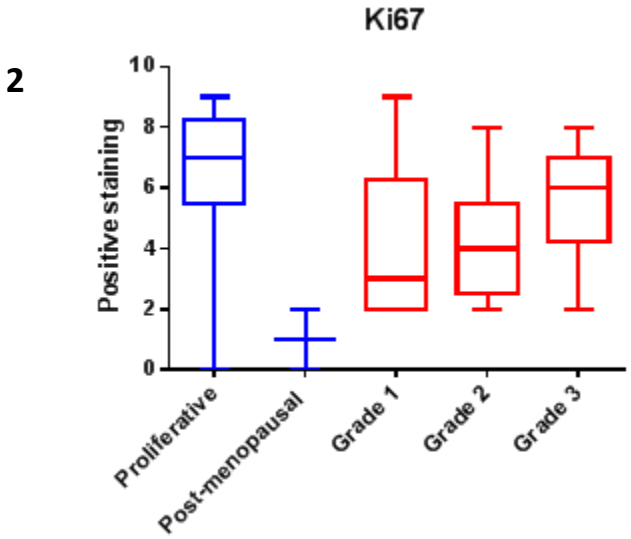

Figure 16: Immunohistochemical staining of Ki67.
1) Immunohistochemical staining for Ki67 of normal proliferative phase endometrium (n=10), post-menopausal endometrium(n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification (scale bar 100µm). B) 400x magnification (scale bar 50µm) .
2) Box plots showing median, inter-quartile range and range for estimated percentage positively stained nuclei for Ki67.

4.2.3 SOX9

SOX9 is known to be associated with the Wnt/β-catenin pathway; SOX9 expression has been reported to be dependent on Wnt signalling, but has also been postulated to be a regulator of the Wnt/β-catenin pathway. SOX9 has been postulated to attenuate Wnt-target gene transcription, this ensuring Wnt-stimulated proliferation is kept in check[70, 71].

SOX9 staining was only exhibited in the epithelial and not the stromal compartment of the endometrium (Figure 17). SOX9 was primarily located in the nucleus, although cytoplasmic staining was also seen, often in conjunction with nuclear staining. SOX9 is a transcription factor; therefore a nuclear location would be necessary to affect transcription of target genes.

Essentially every epithelial cell in PM glands was strongly positively stained for SOX9. Glands in PP samples showed a wider range of staining intensity, but overall there was a considerably lower expression of nuclear SOX9 in the PP samples (MW $p<0.0001$) than PM samples. Malignant samples demonstrated a significant decrease (MW $p<0.0001$) in comparison to PM samples, although there was little variation between grades of cancer or between cancer and PP samples. Interestingly, where glands have retained normal endometrial glandular architecture in the surrounding malignant tissue, SOX9 expression is retained, similar to the normal postmenopausal tissue. It is important to note that for all

proteins, but especially SOX9, that the malignant tissue sections comprised of a very heterogeneous staining pattern.

Despite its putative relationship with the canonical Wnt/β-catenin pathway, expression of nuclear β-catenin and SOX9 were not correlated. There was however a negative correlation between SOX9 and the proliferation marker Ki67 (SR p=0.032, correlation co-efficient -0.300).

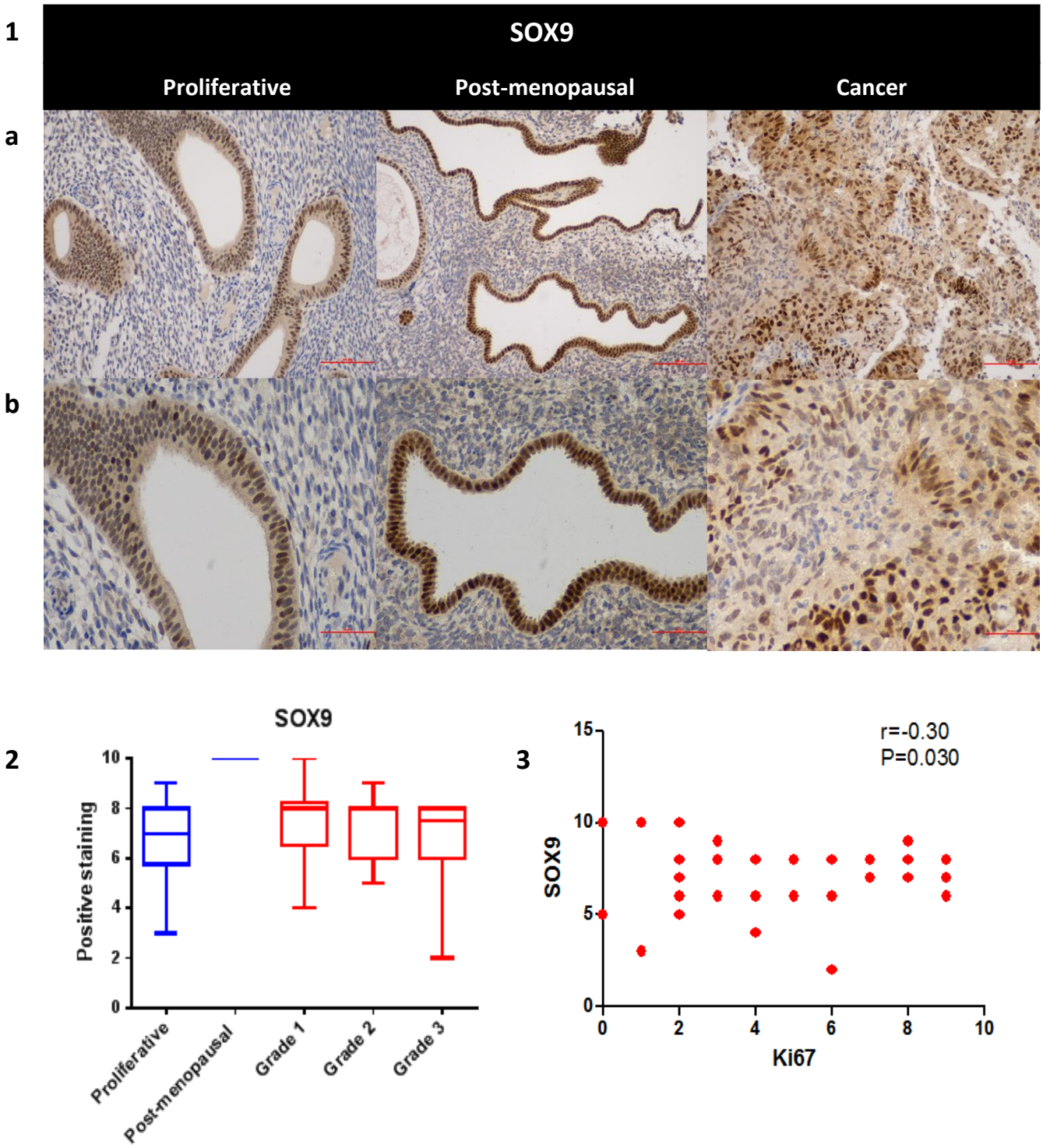

Figure 17: Immunohistochemical staining of SOX9. 1) Immunohistochemical staining for SOX9 of normal proliferative phase endometrium (n=10), post-menopausal endometrium(n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification (scale bar 100µm). B) 400x magnification (scale bar 50µm). 2) Box plots showing median, inter-quartile range and range for estimated percentage positively stained nuclei for SOX9. 3) Scatter graph to show the correlation between SOX9 and Ki67.

4.2.4 SOX2

SOX2 has been proposed as a marker of progenitor cells due to its roles in development, differentiation, pluripotency and the maintenance of a cell's ability for proliferative potential. SOX2 is also implicated in carcinogenesis in a variety of organs, in addition to being proposed as a regulator of the canonical Wnt/β-catenin pathway. The role of SOX2 in the normal and malignant endometrium remains unclear.

The expected localisation of SOX2 would be nuclear due to its role as a transcription factor, although a significant amount of cytoplasmic staining was also seen. Whilst there was a range of both proportion of positive staining and intensity of the cytoplasmic staining between the samples, there was no distinction between sample types (KW p=0.283) (Figure 18). With few exceptions, the proportion of positively stained nuclei was <1% across all groups, consequently there were no inter-group differences observed (KW p=0.285). SOX2 defines an undifferentiated cell; consequently a low expression would be expected. However, of the 7/10 samples which demonstrated >1% nuclear staining and 6/7 samples with >2% positive staining were from malignant samples. All bar one of the samples with >2% staining are high grade cancers. It is possible that the SOX2 expressing cells represent a cancer stem cell. These differences are small, but may suggest that a differential expression could be unmasked with a larger sample size.

Due to its postulated relationship with the canonical Wnt/β-catenin pathway, a correlation was assessed in relation to nuclear B-catenin (SR p = 0.025, correlation co-efficient 0.313).

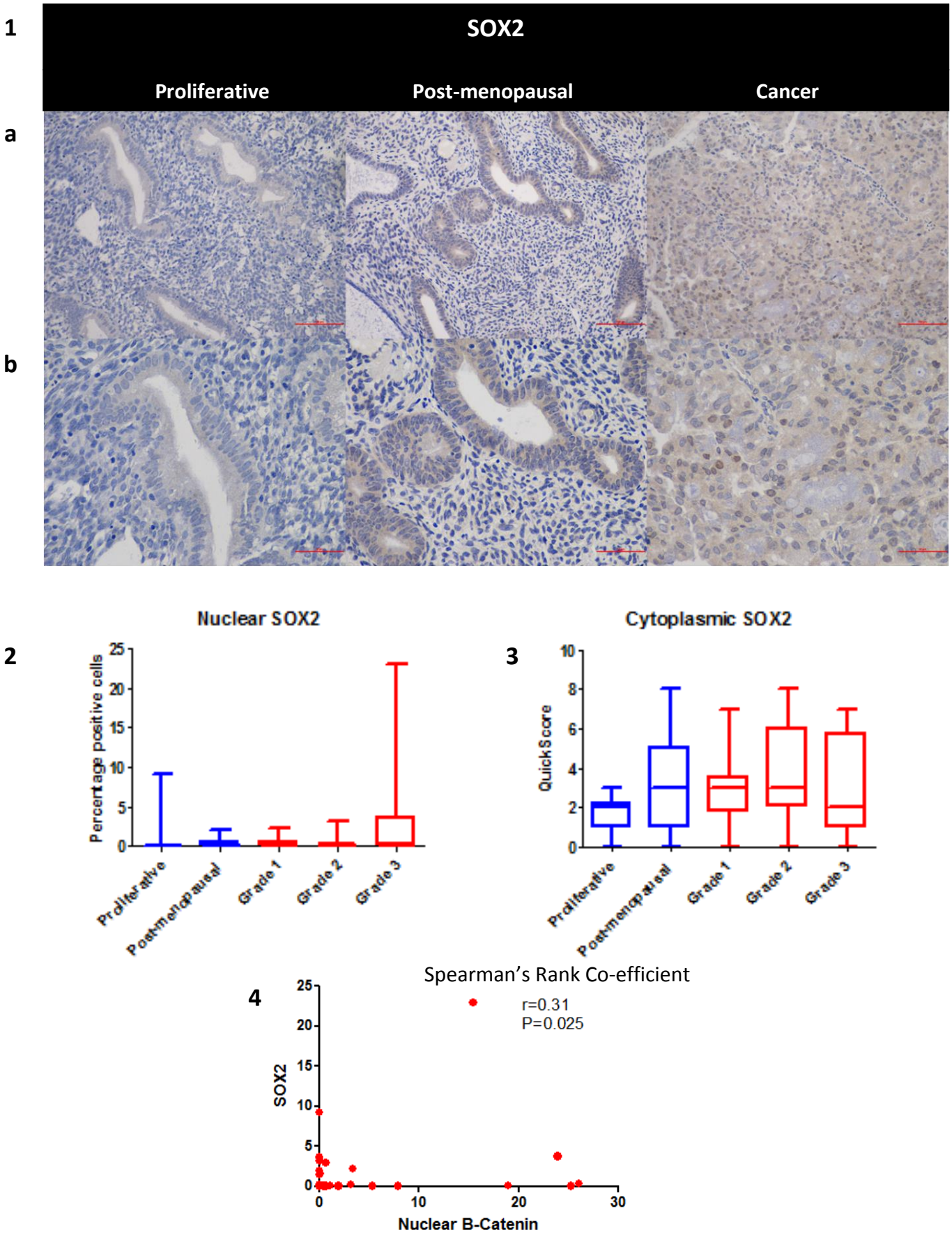

Figure 18: Immunohistochemical staining of SOX2. 1) Immunohistochemical staining for SOX2 of normal proliferative phase endometrium (n=10), post-menopausal endometrium(n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification. B) 400x magnification.
2) Box plots showing median, inter-quartile range and range for percentage positively stained nuclei for nuclear SOX2. 3) Box plots showing median, inter-quartile range and range for QuickScore of cytoplasmic SOX2 staining. 4) Scatter graph to show correlation between SOX2 and nuclear β-catenin.

4.2.5 NAP1L1 and SFRS2

The histone chaperone transporter NAP1L1 and splicing factor SFRS2 were identified in colorectal carcinoma arrays as being up-regulated downstream of nuclear β-catenin in the malignant colon. NAP1L1 has not been reported previously in the endometrium. SFRS2 has been identified in the normal endometrium in the context of implantation. Neither of these proteins has been investigated in endometrial carcinoma.

SFRS2 and NAP1L1 demonstrated positive staining in the endometrial epithelial glands, and to a lesser extent the stroma. Both proteins were expressed in the cytoplasm in addition to the nuclei of the cells. There was no difference in staining pattern or intensity between cancer and either the PP or the PM controls (SFRS2: KW p=0.124; NAP1L1: KW p=0.155) (Figures 19 and 20).

Nuclear β-catenin was positively correlated with NAP1L1 (SR p=0.046, correlation co-efficient 0.281) and SFRS2 (SR p=0.032, correlation co-efficient 0.301) in line with their status as Wnt-related proteins (Figure 21). NAP1L1 and SFRS2 also demonstrated a positive correlation with each other (SR $p < 0.0001$, correlation co-efficient 0.613), giving credence to the notion that they share a regulatory pathway.

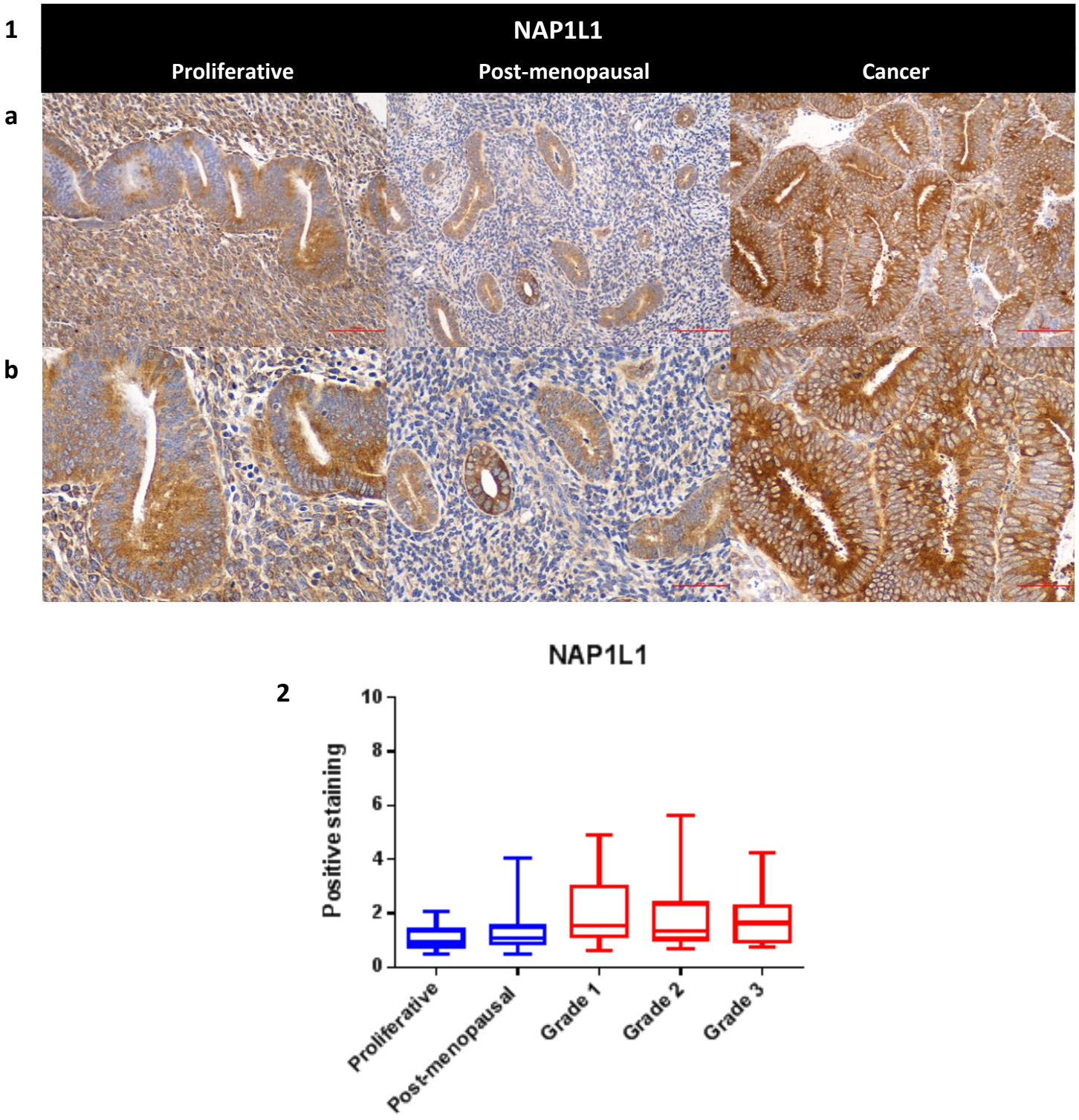

Figure 19: Immunohistochemical staining of NAP1L1. 1) Immunohistochemical staining for NAP1L1 of normal proliferative phase endometrium (n=10), post-menopausal endometrium(n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification (scale bar 100µm). B) 400x magnification (scale bar 50µm).. 2) Box plots showing median, inter-quartile range and range for the positive staining index of NAP1L1. 3) Box plots showing median, inter-quartile range and range for the positive staining index of NAP1L1. As previously described, the positive staining index is derived from the ratio of DAB staining (positive) to haemotoxylin staining using colour deconcolution and area measurement on ImageJ.

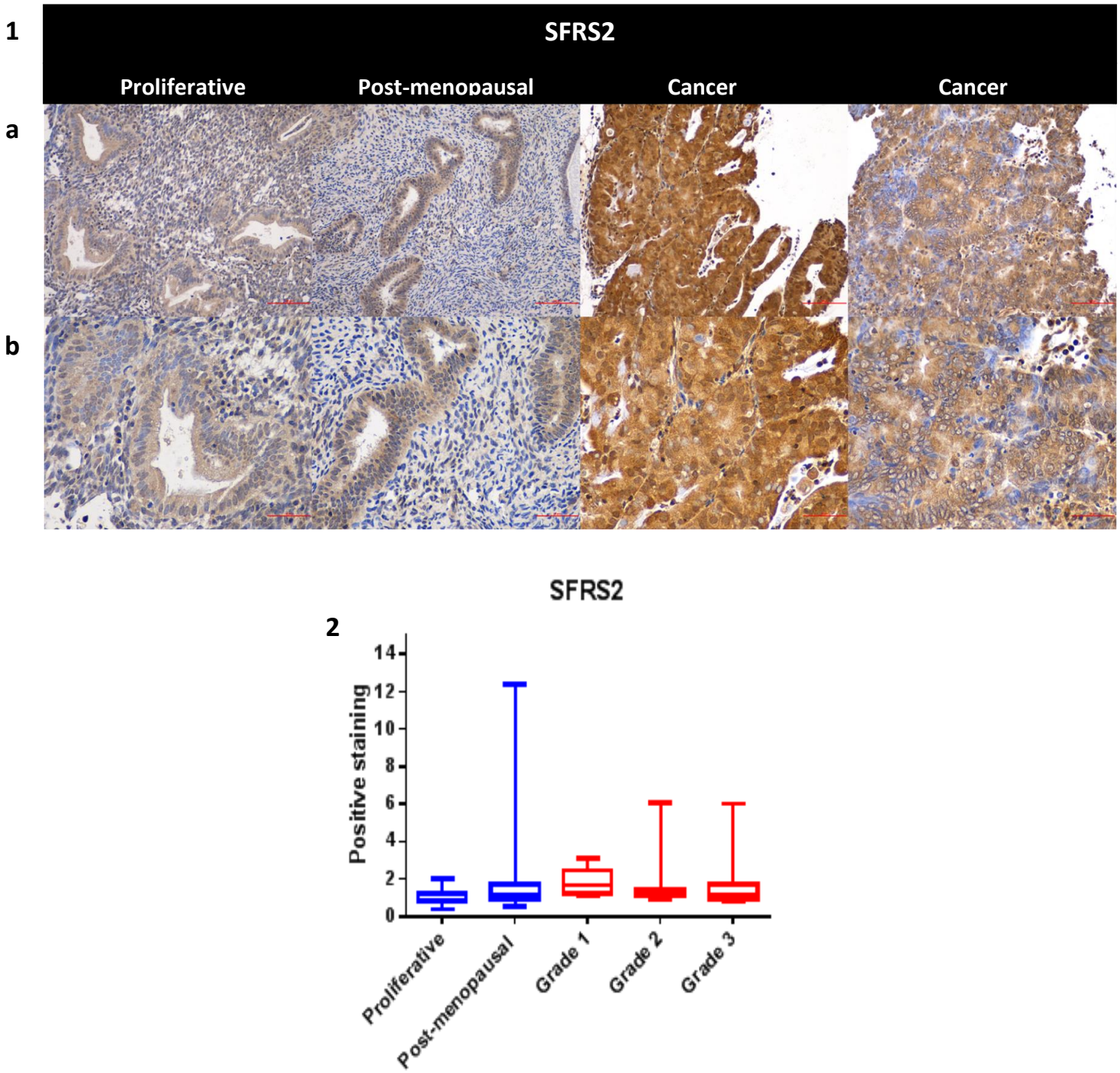

Figure 20: Immunohistochemical staining of SFRS2. 1) Immunohistochemical staining for SFRS2 of normal proliferative phase endometrium (n=10), post-menopausal endometrium (n=10) and endometrial adenocarcinoma samples (G1 n=10, G2 n=12, G3 n=8). A) 200x magnification (scale bar 100µm). B) 400x magnification (scale bar 50µm)..
2) Box plots showing median, inter-quartile range and range for the positive staining index of SFRS2. 3) Box plots showing median, inter-quartile range and range for the positive staining of SFRS2.
As previously described, the positive staining index is derived from the ratio of DAB staining (positive) to haemotoxylin staining using colour deconcolution and area measurement on ImageJ.

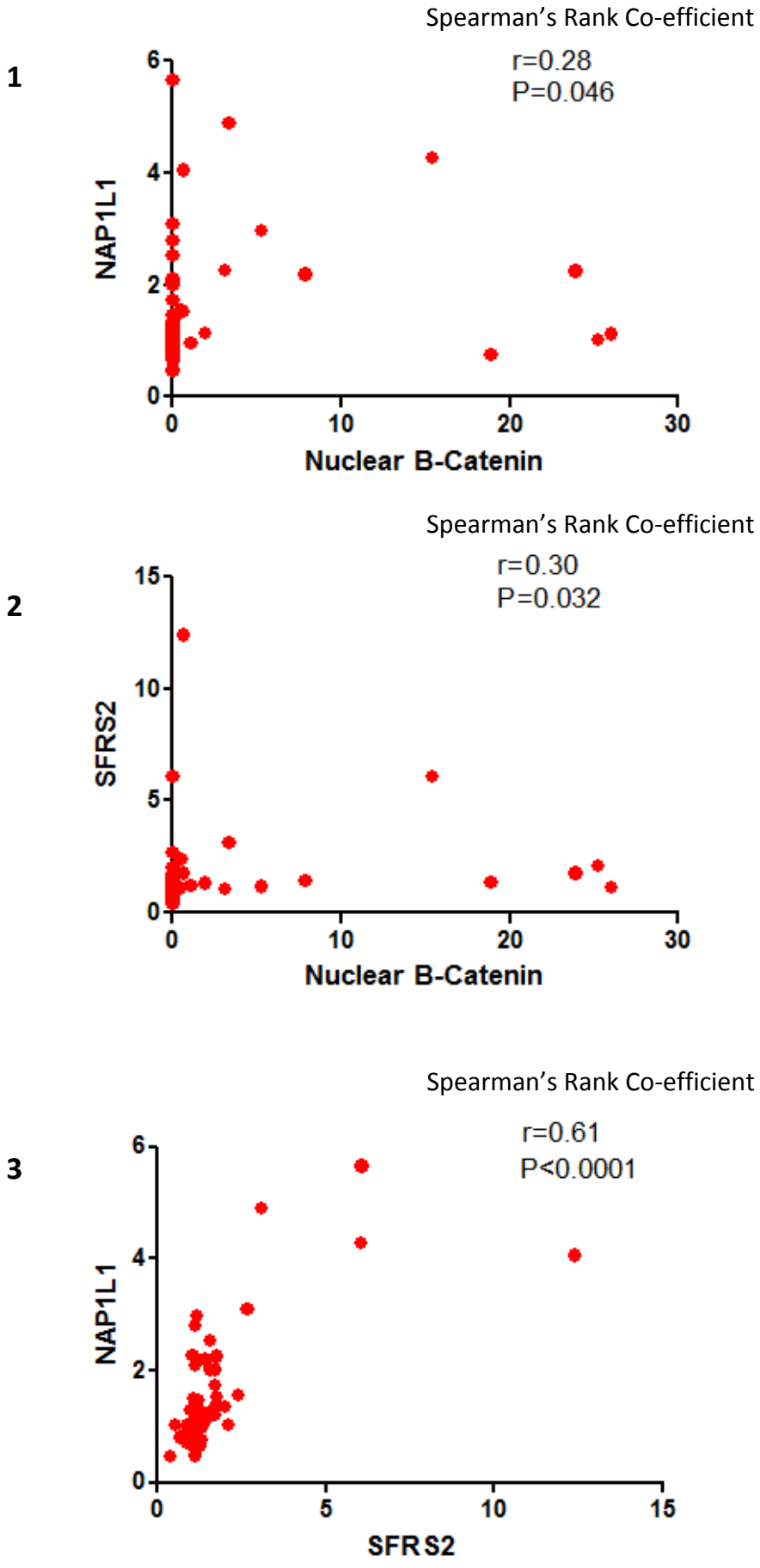

Figure 21: Correlations between nuclear β-catenin and the Wnt-related proteins NAP1L1 and SFRS2. A) Scatter plot to show relationship between nuclear β-catenin and NAP1L1. B) Scatter plot to show relationship between nuclear β-catenin and SFRS2. C) Scatter plot to show relationship between NAP1L1 and SFRS2.

4.3. PCR

Oestrogen and progesterone are key regulators of the human endometrium, both in the healthy and disease state. Their potent effects on proliferation are mediated through their steroid receptors. In malignancy, the balance of these interactions is disrupted and can result in clinical implications. For example, progesterone is a well-tolerated, potent anti-proliferative agent, but its clinical use is limited in endometrial cancer as it is difficult to predict sensitivity[15]. SOX9 is another postulated regulator of cell proliferation through the canonical Wnt/β-catenin pathway. IHC demonstrated a very high SOX9 expression in the atrophic endometrial epithelial glands, with a significantly lower expression in the proliferative tissues.

In order to investigate the effects of ovarian hormones on endometrial cancer, a cell line (well differentiated Ishikawa cell line derived from a grade 1 endometrial adenocarcinoma) was employed. The chosen treatments represent the dominant hormones involved in endometrial regulation. Oestrogen (E_2) was used both alone and in combination with progesterone (a synthetic, highly potent progestagen MPA[106]). Progesterone (MPA) was also assessed in isolation from oestrogen. Since some of the effects of progestagens in the endometrial epithelial cells are thought to be mediated via FGF[15] (basic FGF), this was also assessed. The dominant hormones in the post-menopausal endometrium (the primary location for endometrial carcinogenesis) are androgens (potent AR agonist DHT), despite this, they have not been widely investigated[7].

The mRNA of expression of expression of oestrogen receptor α (ERα), progesterone receptor (PR) and SOX9 were assessed using RT-PCR and compared to untreated cells (vehicle).

The PCR products are shown below after electrophoresis and image capture (Figure 22). mRNA expression of each gene was quantified by densitometry on ImageJ, and normalised to the housekeeping gene YWHAZ. The Kruskal-Wallis test was performed to show differences between the treatments for each gene. Where differences were demonstrated, the Mann-Whitney test was performed to investigate where the differences lie.

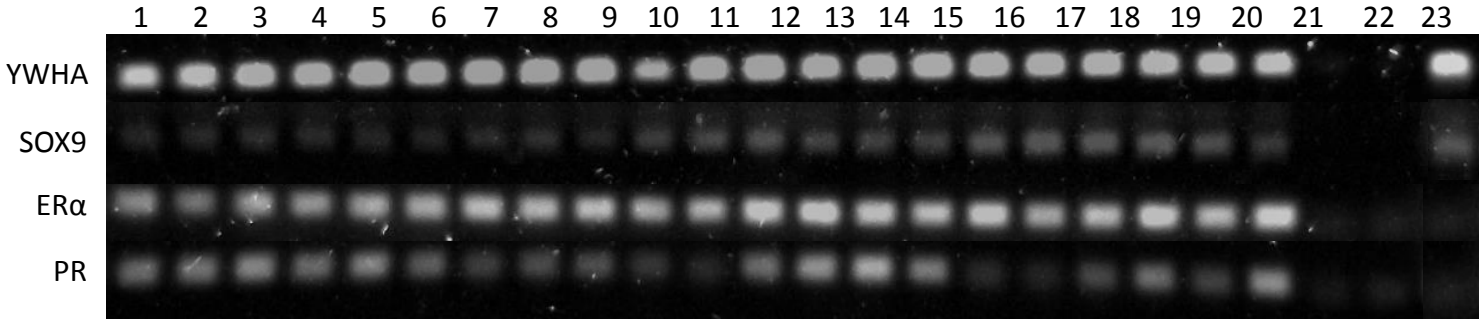

Figure 22: Gel electrophoresis of PCR products

1. E_2	**7.** MPA	**13.** DHT	**19.** Vehicle
2. E_2	**8.** MPA	**14.** DHT	**20.** Vehicle
3. E_2	**9.** MPA	**15.** DHT	**21.** SPCEX123
4. E_2 + MPA	**10.** FGF	**16.** Vehicle	**22.** No template control
5. E_2 + MPA	**11.** FGF	**17.** Vehicle	**23.** No enzyme control
6. E_2 + MPA	**12.** FGF	**18.** Vehicle	**24.** Prostate

4.3.1 Oestrogen receptor α

No difference was noticed between treatments in expression of ERα. (Figure 23, Table 19).

Table 19: Densitometry results for Oestrogen receptor α

Treatment	Densitometry				Normalised Ratio	Mean normalised Ratio
	1	2	3	Average		
E_2	16833.87	16495.04	16601.45	16643.45	0.86	0.69
E_2	8618.75	10049.53	10507.77	9725.35	0.46	
E_2	15446.5	17004.4	15984.74	16145.21	0.76	
E_2 + MPA	13561.26	13524.26	13983.09	13689.54	0.66	0.75
E_2 + MPA	16625.28	16625.28	17742.64	16997.73	0.78	
E_2 + MPA	14212.43	16037.71	16741.54	15663.89	0.82	
MPA	18648.99	18438.99	18126.16	18404.71	0.87	0.88
MPA	17531.45	18505.94	18055.11	18030.83	0.85	
MPA	18810.57	19251.81	19748.64	19270.34	0.94	
FGF	14942.62	15758.28	16031.11	15577.34	1.07	0.94
FGF	14877.92	15896.28	17159.35	15977.85	0.83	
FGF	19834.67	20983.16	21087.16	20635	0.94	
DHT	20677.97	22380.11	21468.45	21508.84	1.09	0.93
DHT	18092.26	18924.21	19360.74	18792.4	0.89	
DHT	17437.69	18606.76	18033.94	18026.13	0.82	
Vehicle	19372.38	20891.28	20278.04	20180.57	0.92	
Vehicle	14655.72	16265.28	15966.87	15629.29	0.74	
Vehicle	17830.16	17838.4	18386.23	18018.26	0.86	
Vehicle	20490.21	20647.62	21085.87	20741.23	0.98	
Vehicle	15800.67	17787.11	17992.11	17193.3	0.88	

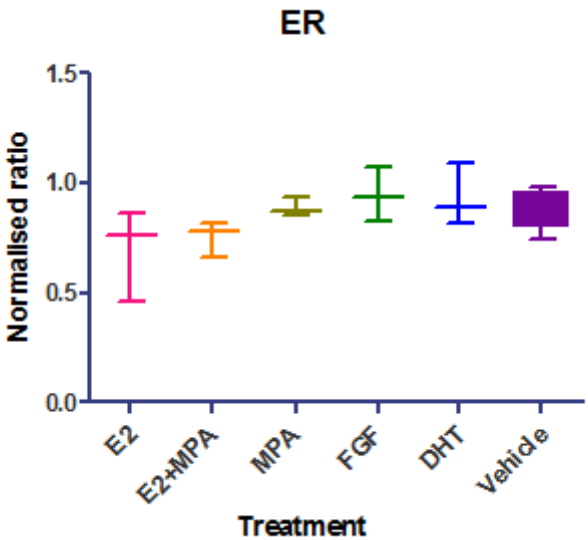

Figure 23: RT-PCR for oestrogen receptor α. Box plots showing normalised ratios of densitometry values for mRNA levels of ERα following 72-hour ovarian steroid hormone treatment.

4.3.2 Progesterone receptor

Progesterone receptor was up-regulated by E_2, E_2+MPA and DHT from the baseline (vehicle). Neither MPA nor FGF altered the expression of SOX9 (Figure 24, Table 20).

Table 20: Densitometry results for Progesterone Receptor

Treatment	Densitometry				Normalised Ratio	Mean normalised Ratio
	1	2	3	Average		
E_2	17730.57	19170.06	19751.3	18883.98	0.97	0.96
E_2	18612.47	19493.42	20424.08	19509.99	0.91	
E_2	20550.76	21735.25	21631.84	21305.95	1.00	
E_2 + MPA	15500.87	17101.42	17458.84	16687.04	0.80	0.78
E_2 + MPA	17456.35	18324.59	19098.25	18293.06	0.84	
E_2 + MPA	13112.04	12975.45	14530.84	13539.44	0.71	
MPA	8657.6	9293.26	9387.84	9112.9	0.43	0.51
MPA	10205.24	10813.48	11953.79	10990.84	0.52	
MPA	11557.97	11788.38	11999.38	11781.91	0.57	
FGF	7973.7	8399.7	8978.94	8450.78	0.58	0.55
FGF	6143.32	6510.97	6887.22	6513.837	0.34	
FGF	15696.94	16563.59	16236.18	16165.57	0.73	
DHT	20443.54	22325.27	20688.96	21152.59	1.07	1.02
DHT	24063.2	25745.34	24741.27	24849.94	1.18	
DHT	17122.94	17899.84	18839.91	17954.23	0.81	
Vehicle	7733.92	7759.51	8313.75	7935.727	0.36	
Vehicle	6263.44	7793.87	7621.87	7226.393	0.34	
Vehicle	11958.64	12984.84	12201.64	12381.71	0.59	
Vehicle	16609.42	16615.42	16721.42	16648.75	0.79	
Vehicle	10492.92	10757.33	11861.52	11037.26	0.56	

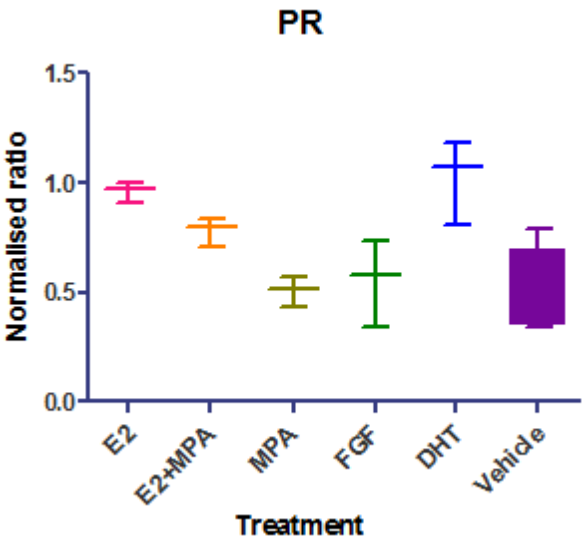

Figure 19: RT-PCR for progesterone receptor. Box plots showing normalised ratios of densitometry values for mRNA levels of PR following 72-hour ovarian steroid hormone treatment.

4.3.3 SOX9

SOX9 was down-regulated by MPA, FGF and DHT in the region of 1.5-fold. Neither E_2 nor E_2+MPA treatment altered the expression of SOX9 (Figure 25, Table 21).

Table 21: Densitometry results for SOX9

Treatment	Densitometry				Normalised Ratio	Mean normalised Ratio
	1	2	3	Average		
E_2	11026.82	9762.05	10285.87	11026.82	0.53	0.45
E_2	9229.75	8284.1	8855.22	9229.75	0.41	
E_2	8980.41	8377.75	8435.46	8980.41	0.41	
E_2 + MPA	8904.87	8869.87	9069.29	8904.87	0.43	0.41
E_2 + MPA	8832.22	8594.8	8438.39	8832.22	0.40	
E_2 + MPA	7507.97	7647.39	7762.39	7507.97	0.40	
MPA	5612.37	6083.61	5939.2	5612.37	0.28	0.30
MPA	6823.02	6531.9	6890.73	6823.02	0.32	
MPA	6614.73	6289.9	6484.73	6614.73	0.32	
FGF	4524.3	4891.54	4383.3	4524.3	0.32	0.31
FGF	6297.07	5941.25	5946.25	6297.07	0.31	
FGF	7011.27	6707.44	6985.56	7011.27	0.31	
DHT	5454.37	5216.42	5341.12	5454.37	0.27	0.30
DHT	5804.95	5284.71	5959.37	5804.95	0.27	
DHT	8171.95	7884.54	8545.2	8171.95	0.37	
Vehicle	7851.15	7469.61	7907.44	7851.15	0.35	
Vehicle	11229.2	12611.39	13373.63	11229.2	0.59	
Vehicle	9252.85	8853.32	9419.85	9252.85	0.44	
Vehicle	9109.44	9249.27	9258.85	9109.44	0.44	
Vehicle	9719.8	9711.8	9557.1	9719.8	0.49	

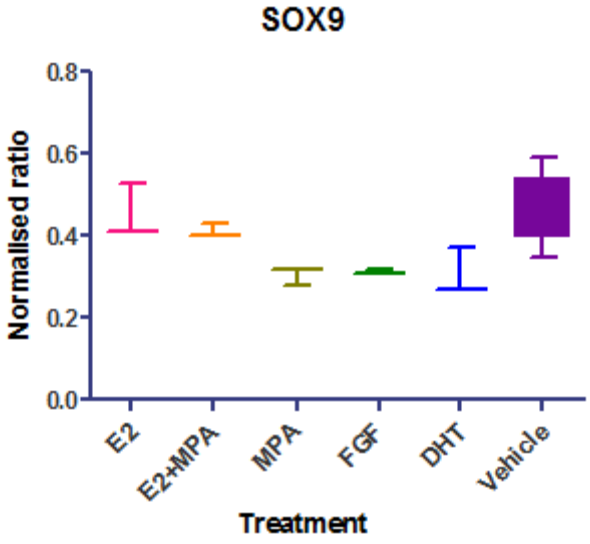

Figure 20: RT- PCR for SOX9. Box plots showing normalised ratios of densitometry values for mRNA levels of SOX9 following 72-hour ovarian steroid hormone treatment.

Chapter 5. Discussion

The canonical Wnt/β-catenin pathway plays a vital role both in the functioning of the normal endometrium, and in endometrial carcinogenesis. Traditionally, the endometrium has been viewed as being dominated by progesterone and oestrogen – both of which can modulate the Wnt/β-catenin pathway. However, endometrial adenocarcinoma is almost exclusively a disease of the post-menopausal endometrium which is by definition hypo-oestrogenic. Investigation of other Wnt-pathway modulators in the endometrium is therefore crucial to further our understanding of this common malignancy. SOX9 has been previously described to limit Wnt/β-catenin induced proliferation[69], has well described interactions with the prostatic androgen receptors[107], and has been shown here to be highly expressed in the androgen mediated atrophic post-menopausal endometrium. The results presented here provide further evidence for the pivotal role played by Wnt in the endometrium, as well as preliminary data on a role for SOX9 and androgens.

5.1. Expression of Wnt-related proteins in primary endometrial samples

Immunohistochemical analysis of the relative expression of the Wnt-related proteins β-catenin, SOX9, SOX2, NAP1L1 and SFRS2, and the proliferation marker Ki67 was performed in endometrial adenocarcinoma, normal proliferative phase and post-menopausal samples. The Wnt/β-catenin pathway is known to have an important role in the normal endometrium as it maintains the balance between stemness and proliferation[108]. Aberrations of this pathway are frequently seen[3]. APC is one of the most common mutations in endometrial cancer[109]; without APC the destruction complex cannot function and enables the Wnt-related proteins to be expressed beyond the control of Wnt. The Wnt-related proteins examined here have all been described in other related cancers, but little is known in the endometrium. The potential implications of the findings for each of the proteins are discussed below.

5.1.1 β-catenin

The canonical Wnt/β-catenin pathway is known to have an important role in regulating normal endometrial homeostasis. During the normal menstrual cycle, high oestrogen levels induce Wnt activation during the proliferative phase. This is counterbalanced by progesterone during the secretory phase which inhibits Wnt/β-catenin signalling, therefore inducing differentiation[3]. Dysregulation of this pathway caused either by hormonal imbalance or mutations are implicated in endometrial carcinogenesis[109].

Immunohistochemical staining of β-catenin reveals a varied pattern of expression in the endometrium, with junctional, cytoplasmic and nuclear accumulation being seen (see Figure 14). As previously discussed, this represents the functional roles of β-catenin, and it is therefore important to consider the intra-cellular compartment in which it lies in order to assess Wnt activation. Transcription of the down-stream proliferative genes, within the benign setting, is dependent on nuclear β-catenin acting as a transcription factor.

5.1.1.1. Nuclear β-catenin expression in the benign endometrium

The benign endometrium demonstrated primarily cytoplasmic and junctional staining, with nuclear β-catenin seen very rarely in the proliferative functionalis. The literature suggests that nuclear β-catenin expression is up-regulated in the proliferative phase[105] of the normal endometrium, which was not seen here. However, these studies rarely differentiate between the functionalis and basalis layers. Where the functionalis and basalis have been regarded separately, the results are similar to that found here[32, 50, 110]. It has recently been reported that nuclear β-catenin is a feature of the basalis layer[12], which in addition to mediating the regeneration of the functional layer is the proposed location of origin of the endometrial proliferative conditions[12].

The basalis of the pre-menopausal endometrium is reported to have a similar gene signature to the post-menopausal endometrium[32]; it is perhaps therefore to be expected that the post-menopausal group displayed increased nuclear β-catenin

compared to the proliferative functionalis. Moreover, the post-menopausal endometrium has been demonstrated to have intact Wnt/β-catenin signalling, although under a negative feedback loop in order to down-regulate transcription of the proliferative genes[75]. The basalis layer of the pre-menopausal samples was not assessed in this study, as the primary aim was to provide a suitable control for endometrial cancer samples which were all pipelle rather than full-thickness samples, and not to characterise the normal endometrium. The limitations of this will be discussed.

5.1.1.2. Cytoplasmic β-catenin expression

The role of cytoplasmic β-catenin is more difficult to distinguish. Activation of the canonical Wnt/β-catenin pathway, results in translocation of β-catenin to the nucleus and subsequent transcription of downstream proliferative genes[111]. Conversely a lack of Wnt activation should result in β-catenin degradation by the destruction complex. It has been proposed that increased cytosolic levels of β-catenin encourage translocation to the nucleus, and therefore some degree of cytoplasmic β-catenin is necessary for Wnt-related proliferation[112]. On the other hand, β-catenin has three potential binding partners, only one of which (TCF/LEF) lies in the nucleus; both the cadherins and Axin/APC are cytoplasmic in origin[112]. It has been suggested that on leaving the ribosome, the first binding partner is cadherin. Only when there is surplus β-catenin than is required for the adherens junctions does it become available to Axin/APC. If Wnt activation occurs, β-catenin can translocate to the nucleus and bind to the third binding partner TCF/LEF,

otherwise, it is degraded by the second[112]. Whilst not formally assessed, junctional β-catenin (bound to cadherin at the adherens junctions), could be seen in the absence of either cytoplasmic or nuclear β-catenin.

5.1.1.3. β-catenin expression in endometrial adenocarcinomas

Nuclear β-catenin was present in approximately 40% of the endometrial adenocarcinoma samples. The range in estimates of Wnt/β-catenin pathway activation as measured by nuclear β-catenin staining is very large, from 16-85%[89, 113]. The number of Wnt-activated samples studies here sits squarely in the middle of these estimates. In the same manner that there is no consensus on the incidence of samples expressing nuclear β-catenin, any association between grade of cancer and expression is equally unclear. This study found that there was no significant difference of nuclear β-catenin expression between the grades of cancer, which has been previously been reported[105, 114]. It must be noted that the samples sizes used here are small, which decreases the likelihood of inter-group differences being significant. There was also no difference in nuclear β-catenin expression between the cancer samples and normal controls. This is somewhat surprising since β-catenin mutations are an early step in endometrial tumorigenesis, and may also be involved in later disease progression[65]. However, when presented graphically (Figure 16) the data shows a clear trend for higher expression in malignant samples. It appears that nuclear β-catenin may be associated with stage rather than grade; low-stage cancers appear to express more nuclear β-catenin than invasive

disease[115]. This was not possible to assess due to not having access to high-stage disease samples.

This study found that cytoplasmic β-catenin was only differentially expressed between both of the normal controls and grade 2 cancers. However, there would appear to be a trend in decreased cytoplasmic staining and increasing grade of cancer; the reverse of the trend for nuclear β-catenin. It could be inferred from this that the cytoplasmic pool of β-catenin is being depleted due to nuclear translocation. However, due to the uncertainty of the implications of cytoplasmic β-catenin outlined above, the utility of assessing cytoplasmic β-catenin is questionable. This is perhaps best demonstrated by the correlation of nuclear β-catenin and the Wnt-related proteins SOX2, SFRS2 and NAP1L1, none of which were directly correlated with cytoplasmic β-catenin.

5.1.1.4. Association between nuclear β-catenin expression and proliferation

The lack of correlation between Ki67 and nuclear β-catenin is perhaps surprising; however, this lack of association has previously been described[97, 116]. There has been a report of a correlation between Ki67 and nuclear β-catenin[117], although this study included endometrial adenocarcinoma patients who had received progesterone treatment. There is a distinct lack of literature which directly analyse the relationship between nuclear β-catenin accumulation and a marker of proliferation such as Ki67 in the endometrium. It is unlikely that the mechanism which regulated the complex nature of proliferation and regeneration in the

endometrium is a simple one, and it is likely that several mechanisms interact. Moreover, in the malignant state there is a high occurrence of aberrations in the Wnt/β-catenin pathway, and Wnt activation in the absence of nuclear β-catenin has been reported in other cancers[112]. Another point to consider would be that malignancies are heteregenous in nature, and nuclear β-catenin staining was not evenly distributed across entire sections. It was not always possible to use consecutive sections from the tissue-containing paraffin blocks, which may affect the correlations between proteins.

5.1.2 Ki67

The Wnt/β-catenin pathway regulates the fine balance between proliferation and differentiation in the endometrium; nuclear β-catenin has been used as a marker of Wnt-activation[105]. Wnt activation is associated with transcription of down-stream genes such as NAP1L1 and SFRS2. In order to assess whether Wnt-related protein expression was associated with proliferation, Ki67 was used.

The post-menopausal endometrium, increasingly recognised as not always being entirely quiescent, shows low-levels of proliferation[7]. This was demonstrated by the Ki67 scores, which were representative of a far lower proliferative index than either the PP or cancer samples. Without exception, the post-menopausal samples displayed low Ki67 staining, although whilst some glands appeared to be entirely quiescent, others showed some low-level proliferative activity. These could

represent the proliferative foci in the active post-menopausal endometrium due to low level oestrogenic activity described by Sivridis *et al.*[33].

That post-menopausal endometria are not all entirely inactive has long been known[118, 119], however, the extent of activity and the risks that this may pose are becoming increasingly known. As previously discussed, Sivridis *et al.*[33] propose that endometrial adenocarcinomas are derived from the glands which demonstrate some proliferative activity in response to low level oestrogenic stimulation in the presence of a functioning ER system. They conclude that the risk of developing endometrial adenocarcinoma from inactive glands is exceptionally low.

The proliferative index of the cancer samples was equal to, if not lower than the PP samples. It could be proposed that the high-grade cancers ought to show significant levels of proliferation, which they do when compared to the quiescent PM tissue. There are several factors to consider when interpreting this result. Firstly, the normal endometrium possesses a remarkable ability to regrow the entire functionalis layer in a matter of a few days, and so it is entirely plausible that the proliferative phase glands express more Ki67 than their malignant counterparts. Secondly, whilst Ki67 expression is strictly correlated with active phases of the cell cycle and cell proliferation, and is one of the most widely accepted markers, it is representative only of a snapshot in time. Ki67 staining will be negative when cells are not in the active cell cycle, and therefore cannot predict proliferative potential. In order to do this a marker such as proliferating cell nuclear antigen (PCNA) would have to be used. PCNA was not included in the staining panel, as its role is not

confined to DNA replication, but is also involved in DNA repair. Additionally, the sections were analysed by scoring ten consecutive high-power fields, which ought to balance the considerable heterogeneity shown by the cancer samples. However, it is possible that it has not accounted for this entirely.

The normal proliferative phase samples showed a considerable range in staining. This could be related to the inter-mitotic period, but it is more likely that there is an intra-phasic variation in proliferation during the phase. For the purposes of this study, proliferative phase endometrium was diagnosed on the basis of histological dating and last menstrual period, and no differentiation was made between early and late phase. It would not be unexpected for levels of proliferation to vary during this period in response to hormone levels, and it is known that later in the phase the glands show increased levels of proliferation[10]. It may be of utility to sub-divide the PP samples into an early and late proliferative phase group by integrating the peripheral hormone profile into the dating of the sample. It must also be noted that discrepancies occur between different dating methods, and it is not possible to control for all variables such as length of menstrual cycle.

5.1.3 SOX9

High expression of SOX9 was identified by the post-menopausal endometrium[97], and it was speculated that it may have a role in maintaining the atrophic state. SOX9 has been shown to be up-regulated or down-regulated in many cancers, thereby suggesting a tissue specific role[68].

The nuclear expression of SOX9 was investigated, although it was noted that where nuclear staining was positive, cytoplasmic staining was invariably also present. One of the postulated functions of SOX9 is that it is a transcription factor; therefore nuclear location would be in keeping with its expected function.

SOX9 was solely expressed in the endometrial glandular epithelium, and not in the stromal compartment. The endometrial progenitor cells are thought to reside in the basalis layer, which is the proposed location of origin of cancer initiating cells, and therefore endometrial adenocarcinoma[12]. The basalis layer persists beyond menopause, therefore retaining the putative progenitor cells in the post-menopausal endometrium[12]. Virtually all post-menopausal epithelial cells were strongly SOX9 positive. Both of the highly proliferative endometrial tissues (PP and cancer samples) demonstrated a significant reduction in SOX9 staining. Moreover, SOX9 was negatively associated with Ki67 expression. This may suggest that SOX9 has a role as a marker of low proliferative activity in the endometrium.

To date, there has only been one published report of SOX9 in the malignant endometrium[67]. The results presented by Saegusa *et al.*, show conflicting relationships proliferative index and SOX9. Their immunohistochemistry results demonstrated a positive relationship with Ki67, which was contradictory both to the proliferative profiles shown in their transfection studies, and to the results presented here. Stable over-expression of SOX9 in the HEC251 endometrial cancer cell line by transfection resulted in a decrease in cell proliferation.

There are several key differences between the data presented by Saegusa *et al.* and that described here. Firstly, the study group consisted of 55 endometrioid endometrial carcinomas, of which only 23 were post-menopausal women. Due to the considerably different conditions between the normal cycling endometrium and the atrophic post-menopausal endometrium, the mechanisms of carcinogenesis are likely to be different[106]. This selection of samples is therefore unlikely to be a representative population, since 93% of cases are diagnosed in women aged over 50[115]. One of the key limitations of this paper is the use of proliferative phase endometrium as the positive control. As previously discussed, the vast majority of endometrial adenocarcinomas arise from the post-menopausal endometrium; therefore it would be inappropriate to form a conclusion on the relative up- or down-regulation of a protein without first considering the post-menopausal endometrium. In fact Saegusa *et al.* report a significant up-regulation in endometrial hyperplasia; this may point to loss of SOX9 during carcinogenesis, since hyperplasia is an intermediary between the normal post-menopausal endometrium and malignancy.

The types of biopsy used, and the methods of analysis are slightly ambiguous. Firstly the authors do not state whether the benign samples were pipelle or full-thickness samples, or comment on whether the basalis and functionalis layers were considered separately. It is very difficult to determine what is classified as 'normal' in the human endometrium, due to the wide variation in menstrual cycles and symptoms, and the use of drugs which affect physiology and morphology; no details of exclusion criteria were given.

In addition to this there were also potential methodological differences between the studies. Saegusa *et al.*. stated that the stroma did not express SOX9, but it is unclear whether or not stromal cells were included in the labelling indices, as the references given for the methodology do not include benign samples. The results appear to show that 10% of PP cells are SOX9 positive; the images appear to show considerably more positive epithelial cells than this. The PP samples stained showed considerable variation in staining (Figure), since both studies used a sample size of 10, it is possible that this accounts for the difference. On the other hand, as Saegusa *et al.* used microwave antigen-retrieval, it is possible that the sections show a falsely low expression.

The differences displayed between these studies highlight the need for further investigation of SOX9 in the normal and malignant endometrium.

The prostate is an androgen influenced malignancy, which also has a well described relationship with SOX9. Wang *et al.*[107] have demonstrated that SOX9 contributes to

both decreased cellular proliferation and regulation of the androgen receptor to which it can specifically bind. SOX9 can modulate both expression and activity of prostatic AR. SOX9 expressing basal epithelial cells are thought to support the development and maintenance of the prostatic luminal epithelium, and a subset of prostate cancer cells may escape basal cell requirements through SOX9 expression. Huang *et al.*, have furthered this by suggesting that by maintaining a prostate epithelial lineage which is responsive to oncogenic stimuli, SOX9 permits the initiation of prostate carcinogenesis[120].

The role of SOX9 in maintaining stem/progenitor cells in other tissues such as the intestine, liver, pancreas and hair follicles has been implicated in the pathogenesis of several cancers[121,122].

An activated Wnt pathway has been demonstrated in the post-menopausal endometrium[32], however, this tissue remains relatively quiescent. SOX9 loss in the intestine causes both hyperplasia and dysplasia in the glandular epithelium[70]. In addition there was up-regulation of Wnt target genes. It would not biologically implausible to propose that SOX9 is one of the mechanisms by which Wnt-induced signalling was inhibited in the post-menopausal endometrium. Loss of SOX9 may release this anti-proliferative effect resulting in carcinogenesis.

5.1.4 SOX2

SOX2 has previously been investigated in the normal, endometriotic and malignant endometrium due to its numerous postulated roles. It has long been accepted as a marker of embryonic stem cells, but has also been associated with the canonical Wnt/β-catenin pathway and implicated in carcinogenesis[102].

Nuclear SOX2 was expressed at a low-level across all the study groups. For the vast majority of samples, both benign and malignant, expression was <1% of all epithelial cells. There would appear to be a (non-statistically significant) increase in the grade 3 carcinoma group. Of the 10 samples which expressed >1% positive cells, 7 of these were malignant samples. The proportion of malignant cells with even higher expression (>2%) was 6/7 samples. Expression of SOX2 represents a stem-like cell, therefore it is to be expected that few cells were SOX2+; the SOX2+ malignant cells may be representative of a cancer stem cell.

The reports of SOX2 in the endometrium are very contradictory. The range of reported expression spans from sparse distribution in both benign[100] and malignant endometrium[99, 123]. Some reports suggest that SOX2 expression is in accordance with the stem-like cell markers OCT4 and NANOG[123], while other reports that its expression is 60 times lower[95]. SOX2 has also been reported to be absent in the human endometrium[101]. The low level of nuclear expression reported here, is therefore in accordance with the current literature base. SOX2 has been reported to be essential to maintaining proliferative potential, and together with NANOG

forms part of the transcriptional regulatory circuitry for pluripotency and self-renewal. However, the actions of SOX2 are not autonomous, and must bind to other factors such as OCT4 in order to regulate transcription[93].

It was not possible to pre-power the sample sizes, due to the minimal published data on endometrial SOX2 expression. Therefore increasing numbers of samples may unmask an underlying differential expression. The relevance of cytoplasmic SOX2 remains unclear. There was significant variation in both area and intensity of cytoplasmic staining, although this appeared to be independent of the sample group. In the Wnt/β-catenin pathway setting, SOX2 acts as a transcription factor, so it is likely that the nuclear expression of SOX2 is the most relevant. The differential expression of cytoplasmic SOX2 may be related to another function of the protein. It is also possible that the cytoplasmic staining here is due to a lack of antibody specificity, possibly due to splice variants, as is the case in OCT4[124].

The SOX family of transcription factors have increasingly been identified as key regulators of β-catenin/TCF activity in a variety of biological contexts[69]. SOX2 is a member of this family, and has therefore been investigated as a regulator of the canonical Wnt/β-catenin pathway. Closely related to TCF proteins, SOX proteins can act as either activators or repressors of Wnt signalling[69]. One common feature of the Wnt pathway are negative feedback loops[111], of which SOX2 appears to be one. In addition to stimulating β-catenin activity SOX2 has been reported to be directly regulated by Wnt[69]. In concordance with this hypothesis, nuclear SOX2 was correlated with nuclear β-catenin. Although this would appear to confirm its status

as a Wnt-related protein, there must be some caution, as this correlation is reliant on one sample in this data set. Further investigation with a larger sample size is therefore necessary. It is also beyond the scope of this study to establish whether SOX2 is an up-stream regulator of Wnt in addition to being a target gene.

The role of SOX2 in malignancy is not fully understood, and it seems to act in a tissue-specific manner. For example, SOX2 expression in breast cancer is associated with aggressive properties due to induction of β-catenin induced proliferation[69, 125]. Conversely, SOX2 is often down-regulated in gastric cancer, but when present represses proliferation[92]. In the endometrium, hypermethylation of SOX2 has been reported to be linked to histological subtype, being more common in Type II endometrial carcinomas (non-endometrioid)[96]. SOX2 methylation also appears to be contradictory as a prognostic marker; it has been reported to both be associated with a shorter survival, and with expression of PR (more commonly considered to be a marker of good prognosis)[96]. On the other hand, SOX2 mRNA has been observed to be greater in endometrial tumorosphere cells than differentiated cells[99]. Given the paucity of corresponding results between study groups, further investigation is required before consensus on a role for SOX2 in the endometrium can be reached.

5.1.5 NAP1L1 and SFRS2

Unpublished data (Jenkins *et al.*) reports that NAP1L1 and SFRS2 are up-regulated downstream of β-catenin in colorectal carcinoma. Both proteins are inducible via APC knock-out, and show differential expression between malignant polyps and adjacent normal tissue in both the mouse and human colon. SFRS2 has also been reported in the mouse, primate and human endometrium using immunohistochemistry, RT-PCR and Northern blotting [84, 91, 126]. There have not been any previous reports of NAP1L1 in the endometrium.

The staining patterns of SFRS2 and NAP1L1 appear to be similar to that already reported in both the colon and endometrium. Neither SFRS2 nor NAP1L1 displayed any inter-group variation in expression by analysis using colour deconvolution and area measurement. Subjectively, it had appeared that malignant samples typically had a higher intensity of staining. Colour deconvolution is a very useful analysis tool as it is entirely objective, but it is limited in its ability to differentiate between staining intensities. Whether a difference in staining intensity could be of use when both proteins express some degree of cytoplasmic staining ubiquitously is hard to define. One potential method for bypassing this problem would be to quantify NAP1L1 and SFRS2 expression using PCR. In retrospect, a QuickScore may have been a more appropriate scoring method.

SFRS2 and NAP1L1 were both correlated to nuclear β-catenin expression, as well as being correlated to each other. The first correlation appears to support the

hypothesis that they are down-stream proliferative proteins of the Wnt/β-catenin pathway. The second may suggest that they are both regulated by the same means i.e. by Wnt/β-catenin pathway.

The vital role that the Wnt/β-catenin pathway plays in the maintenance of the normal endometrium, outlined previously, is well documented. It could therefore be expected that these two down-stream proteins would be expressed in all the study groups. The expression of nuclear β-catenin was not statistically different between the groups (other than PP and grade 1 cancers). The expression of these Wnt-regulated proteins could reasonably be expected to mirror that of β-catenin.

5.2. The effect of hormonal treatment on the transcription of SOX9, Oestrogen Receptor α and Progesterone Receptor.

The immunohistochemical analysis of primary endometrial samples demonstrated that SOX9 is down-regulated in endometrial adenocarcinoma compared to normal post-menopausal controls at the protein level. In order to perform a functional analysis of SOX9, a well-differentiated endometrial adenocarcinoma cell line (Ishikawa) were grown in phenol red-free media with charcoal stripped serum, prior to treatment with ovarian steroid hormones for 72hrs.

The treatments were chosen due to their known importance in the normal or malignant endometrium. Oestrogen (E_2) is well established as the single most important risk factor for developing endometrial cancer, as well as being instrumental in its maintenance[30]. Progesterone on the other hand has important anti-proliferative effects and is used as an adjuvant therapy in treating high grade or stage disease[15]. The effects of progestagens are mediated by FGFs released by the stroma[15]. Since the Ishikawa cell line represents a mono-cellular system, and lacks the stromal signalling pathways FGF was used as a separate treatment to account for this. Androgens are the most abundant circulating steroid hormone in the post-menopausal endometrium[6]. Local and circulating androgen production has been linked to endometrial hyperplasia and malignancy; significantly elevated testosterone levels have been found in endometrial carcinomas[34]. It has even been postulated that there is a possible direct role for androgens in endometrial carcinogenesis[45, 127].

5.2.1 Oestrogen receptor α

The proliferative effects of endogenous oestrogens are mediated by the oestrogen receptors ERα and ERβ, although in endometrial carcinogenesis ERα is thought to be the primary mediator[18]. Reduction in levels of ERα in endometrial adenocarcinomas are related to increased epithelial-mesenchymal transition and poorer prognosis[128]. Hormone receptor status and response to therapy has been widely discussed[128, 129]; it appears that personalised therapy based on steroid receptor status may provide a solution to what is currently not a particularly effective therapeutic option[130]. Furthermore, the development of an adjuvant therapy which is capable of inducing/suppressing steroid receptors as appropriate could significantly improve the outlook for patients dependent on non-surgical treatment.

The expression of ERα was not altered by any of the treatments in this experiment. This is not entirely consistent with the literature which suggests that Ishikawa cells retain their ability to auto-regulate their hormone receptors in response to E_2. There are several possible explanations for this. The response of ERα to oestrogen is the most widely investigated of the hormone treatments, and although most studies have been able to show ERα up-regulation in response to oestrogen exposure, the response shown appears to be duration dependent. It is likely that ERα has a fast response to oestrogen exposure; up-regulation has been described as early as 7 hours post-treatment (although studies at 3 hours showed no effect[131]). It is therefore possible that by assessing ERα status at 72 hours, the effect has passed. Johnson *et al.*[132] demonstrated that whilst ERα expression increased during

their 24h culture, the effect was not further increased by oestrogen treatment. Given the lack of consensus in the literature, a further investigation to assess expression at multiple time-points would be beneficial. This time-point was appropriate for the other proteins (PR and SOX9).

Progesterone treatment (MPA and FGF) could also have been expected to down-regulate ERα. One explanation for non-responsiveness to treatment is that the different populations of Ishikawa cells have gained different mutations which affect their ability to respond to treatment. Characterisation of the Ishikawa cell line using immunofluoresence in our laboratory (results not presented), show high levels of both ERα and Ki67 staining. This may suggest that ERα is already expressed at its highest level, so it is not possible for expression to be increased. If this activation is genetic mutation driven, it would therefore be unlikely that hormonal treatments would alter this activation. The effect of DHT treatment on the normal or malignant endometrium has not been established, therefore it is possible that DHT may not alter ERα expression.

5.2.2 Progesterone receptor

In the normal cycling endometrium, progesterone exerts anti-proliferative effects via the steroid hormone receptor PR and FGF signalling. Attempts to exploit this mechanism in endometrial adenocarcinomas so far have not been particularly successful. Progesterone treatment is used in a palliative setting; however, a recent Cochrane review casts doubt on its efficacy[130]. This is partly due to difficulties in predicting hormone sensitivity, although recent investigations suggest the use of stromal may emerge as a reliable biomarker[48]. One future challenge is to re-sensitise hormone refractory cancers to hormonal therapies.

In contrast to ERα, PR responded to the ovarian hormone treatments in the Ishikawa cells. PR was up-regulated by oestrogen treatment, which is in keeping with the literature[133]. When MPA was added to oestrogen to provide a combination treatment, the overall effect was again an increase in PR expression, although to a lesser degree than to oestrogen alone. On the other hand, progesterone did not have any effect on the expression of its own receptor. This would suggest that when given in combination, oestrogen provides the dominant influence on the Ishikawa cells. It has been demonstrated in endometrial carcinoma, that regardless of epithelial PR status, progesterone will only have an effect if stromal PR are present and functional[15]. In the normal endometrium, progesterone relies on FGFs being released by the stromal cells in order to regulate endometrial cells. The Ishikawa cell line lacks any stromal cells, which may explain why it does not respond to MPA. However, in order to compensate for this we also

treated with FGFs which did not have an effect on PR levels either. FGF receptor status was not characterised prior to treatment, so it is possible that the Ishikawa cells lack the appropriate receptor in order for PR to respond to treatment. On the other hand, the resistance of progesterone receptor to regulation by MPA may be due to an aberration in hormonal control which is a key feature of endometrial carcinogenesis.

DHT which has not been widely investigated in the context of the endometrium was also noted to up-regulate PR, which has not previously been reported in Ishikawa cells. Androgen treatments can be administered systemically, and may prove to be a promising area of future research as an adjuvant therapy.

5.2.3 SOX9

SOX9 was found to be up-regulated in the atrophic endometrium of the PM samples when compared to the highly proliferative PP and adenocarcinoma samples. Interactions between SOX9 and the ovarian steroid hormones are not understood in the endometrium, although array data points to oestrogen-induced SOX9 down-regulation[75]. One key similarity between the PP and cancer samples other than the decreased SOX9 expression is the dominance of oestrogen. However, the treatment of the Ishikawa cells with E_2 did not have an effect. In fact, the combined treatment of E_2+MPA produced a slight decrease in SOX9 transcript, suggesting that in a similar fashion to the expression of PR, oestrogen is the dominant factor in the combination.

The expression of SOX9 transcript was down-regulated by DHT; androgen induced modulation of SOX9 expression could be expected considering their well described interactions[107, 121]. On the other hand, both the progesterone related compounds MPA and FGF also down-regulated SOX9. There have not been any previous functional studies assessing the response of SOX9 transcript or proteins levels to hormonal treatment in the endometrium published. However, if as postulated, SOX9 in normal benign cells reduces proliferation, it would be expected that MPA would up-regulate SOX9 since it is a potent anti-proliferative agent in the endometrium. The Ishikawa cell line is well-differentiated, so it could be expected that normal regulation is preserved. However, that was not what was seen here. In endometrial adenocarcinoma, the canonical Wnt/β-catenin pathway is known to be

often dysregulated. It is therefore possible that the mechanism by which SOX9 interacts with pathways such as Wnt is disrupted in malignancy. It could even be suggested that dysregulation of the pathway as seen in normal cells may cause paradoxical up-regulation in cancer cells; SOX9 has been reported as both an inhibitor and promoter of proliferation.

The in vitro model used here may also be a limitation. For example, if the protein level is increased, but has a long half-like, by 72 hours, the mRNA may not be increased. In order to assess this, Western blotting would be required. The cell culture method here was 2D, and although the Ishikawa cell line is well characterised, it is possible that it does not respond in the same way that primary cancers do in vitro.

The results presented here are only a preliminary investigation into the effects of ovarian steroid hormones on SOX9. Further studies are required into the functional roles, interactions and regulation of SOX9.

5.3. Limitations

The results of the immunohistochemical characterisation were limited by multiple factors. Primarily the sample sizes which were chosen for feasibility of collection and analysis during the one year project may mask differential expression of the Wnt-related proteins. In addition, as this was novel research, it was not possible to determine a clinically useful difference and therefore pre-power the sample sizes. Moreover, the quantification methods required the use of non-parametric statistical tests which reduces the strength of the evidence, and are more likely to produce a non-significant p-value.

Determining an appropriate control for comparison to the malignant samples was not entirely straightforward; consequently neither of the controls used (PP and PM normal endometrium) provide a perfect comparison. The appropriate control could be considered to be endometrial tissue which is under the influence of oestrogen, which in turn is the primary known regulator in endometrial cancer development and maintenance, such as proliferative phase samples. On the other hand, the vast majority of Type 1 endometrial cancers, including all of the malignant samples used in this study occur in post-menopausal women. However, post-menopausal samples lack proliferative potential. To further complicate matters, only pipelle samples were available for the malignant samples. For this reason only the functionalis layer of the proliferative phase samples was assessed as the basalis is not collected by a pipelle. However, the post-menopausal endometrium is most similar to the pre-menopausal basalis layer which was not assessed. It may be

expected that the malignant samples retain features of the post-menopausal endometrium, and therefore the basalis. Despite this, it would not be appropriate to discard the proliferative samples as otherwise changes seen in the normal proliferative endometrium could be mistaken for malignant variations. For these reasons, both proliferative phase and post-menopausal samples were used as controls. The Wnt down-stream proteins, although not formally assessed, did not appear to have a significant difference in expression between the two layers. On the other hand, both the β-catenin and SOX9 expression is reported to vary significantly between the layers[12]. It may have been appropriate to use laser capture to isolate benign tissue within the sample as the normal control. However, this has the disadvantage that whilst it may appear morphologically normal, the cells may have biochemical and genetic derangements which cannot be seen macroscopically and therefore are not truly 'normal' either. Whilst every effort was taken to ensure that an appropriate normal control was used, these factors must be considered when interpreting the results.

No stage IIIb or IV cancer samples were available for analysis. Samples were collected from patients undergoing hysterectomy, however, those diagnosed with metastatic disease are usually initially treated with chemo- or radiotherapy. Samples collected from this patients would not be suitable, as these treatments alter the morphology and functioning of the tumours.

Only one housekeeping gene (YWHAZ) was used to normalise the expression of the target genes in the PCR experiments. Whilst it has been reported to be the

housekeeping gene of choice for the endometrium[104], the results would be more robust if two genes were used. Moreover there were three biological repeats of the hormone treatment experiment, but technical repeats of the RT-PCR would improve the strength of the data. Furthermore the use of quantitative RT-PCR would increase the accuracy of the reading and provide a quantitative rather than a semi-quantitative output. In addition, as previously discussed, in order to fully assess the expression of ERα it may be appropriate to repeat the experiment to create a time-response curve.

In order to fully assess the effect or non-effect of the steroid hormones, it would be necessary to fully characterise the receptor status of the Ishikawa cell line. Moreover there are further complications to consider when assessing the effect of the treatments. For example, MPA is capable of binding to PR, AR as well as the glucocorticoid receptor, whilst having only a weak affinity of ER. In order to fully block the effects of oestrogen, a specific oestrogen blocker would be required rather than relying on competitive blocking by MPA. There were also limitations with the PR primer used as it could not discriminate between the two PR subtypes (PRa and PRb) which are both expressed by Ishikawa cells, therefore could only give a global picture of total PR levels. Furthermore, the results presented here only represent the Ishikawa cell line's response to these specific conditions. The functional analysis of SOX9 would therefore benefit from the use of multiple cell lines, 3D rather than 2D cell culture and an increased range of both treatment concentrations and combinations of hormones. Furthermore it would be beneficial

to investigate the effects of treatments at the protein levels using Western blots in addition to the mRNA level.

5.4. Further work

Numerous questions arise from the data presented here, primarily regarding the functional assessment of SOX9. The number and combination of hormonal treatments as well as means of quantification were limited by the timescale of this project. Since DHT appeared to diminish the expression of SOX9, it may be beneficial to perform a dose-response curve to establish whether a higher concentration may have an even greater effect. This is a particularly interesting avenue of investigation as androgens are the dominant hormone in the post-menopausal endometrium, and it is in this phase that SOX9 expression dramatically increases. It would not be biologically implausible for androgens to mediate this change, and may allow the therapeutic exploitation of the anti- proliferative effects of SOX9.

The other treatments which reduced SOX9 expression were both MPA and FGF, it would equally be interesting to investigate whether either of these would act as an adjuvant with DHT. Another combination treatment to consider would be using E_2 and DHT together.

In order to ensure the feasibility of this project, only one cell line was assessed. However, it may be beneficial to extend this study and to use endometrial cancer cell lines which possess different characteristics such as grade, invasion and metastatic potential and hormone receptor status. For example Hec1a and MFE280 which are less well differentiated and demonstrate a greater invasive ability could be used. Another possibility to expand this investigation would be to use xenografts from primary endometrial adenocarcinoma samples.

SOX9 expression in the primary samples was negatively correlated with the proliferation marker Ki67. It is highly expressed in the largely quiescent post-menopausal tissue, but is down-regulated in both the highly proliferative tissues studied. Whether SOX9 is the causative feature of low-proliferation, or a down-stream protein in an anti-proliferative pathway is yet to be ascertained. A logical next step to assess the functional role of SOX9 in vitro would be to switch on/off SOX9 and evaluate the subsequent effect on proliferation, expression of Wnt-related proteins and invasiveness as demonstrated in explant cultures. Furthermore the effect on SOX9 if cells were treated with chemotherapeutic agents could be assessed. In view of the regulatory effect that SOX9 appears to have on the androgen receptor in the prostate, this would be an intriguing line of enquiry in the endometrium.

SOX9 has also been investigated as a prognostic marker in other cancers including breast cancer[134]. It would be possible to collect both survival and outcome data for the samples which have been characterised for their SOX9 expression here, which

could provide an interesting extension of possible SOX9 functions. Furthermore, endometrial cancer has an acknowledged pre-malignant stage. It would therefore be of benefit to assess SOX9 status in primary hyperplasia samples.

5.5. Clinical relevance

The Wnt/β-catenin pathway is acknowledged to be crucial in endometrial carcinogenesis, and these findings would appear to support this. However, the Wnt/β-catenin pathway is also vital for normal homeostasis of the endometrium, and therefore could not be directly therapeutically targeted. In consideration of the poor clinical outcomes for advanced disease, which have shown little improvement in recent years, the lack of effective non-surgical therapeutic options, and the trend of increasing incidence, novel therapies need to be established.

It is being increasingly accepted that not all post-menopausal endometria are equal when it comes to the risk of endometrial carcinoma, and that there is a higher risk group of women with active endometrial glands, about which we know little. Moreover, the only hormones to have been properly considered in the context of endometrial regulation are oestrogen and progesterone. Endometrial cancer is not a disease of the cycling endometrium, there is a need to investigate the dominant hormone in the post-menopausal state – androgens. Expanding knowledge in this area is vital for furthering our understanding of endometrial carcinogenesis. In addition, given the lack of efficacy of the hormonal treatment currently used, this may open a way for a novel treatment for endometrial cancer.

High-dose progesterone treatment is frequently used in the management of advanced disease, but it is limited both by the loss of progesterone receptor and the dysregulation of the progestagen signalling pathways. Whilst it would not be appropriate to use oestrogen to up-regulate PR, DHT also increased the levels of PR transcript in the Ishikawa cell line and could be considered for further investigation as an adjuvant to MPA treatment.

The expression pattern of SOX9 also lends itself to investigation as a potential diagnostic marker in post-menopausal women. Characterisation of the hyperplastic endometrium would be required. Similarly, if SOX9 does have an inhibitory effect on proliferation, it may be possible to exploit this in the management of endometrial cancer.

5.6. Conclusions

The canonical Wnt/β-catenin pathway has a crucial role in regulating the normal endometrium, as well as the transition to malignancy. The transcription factor β-catenin was not significantly increased in malignancy, although there is a trend for increasing Wnt-activation in high-grade disease.

The results here represent the first identification of the Wnt-related proteins NAP1L1 and SFRS2 in the malignant endometrium, and NAP1L1 in the benign endometrium. The expression of NAP1L1, SFRS2 and SOX2 was not different in the

malignant compared to the benign endometrium. However, expression was correlated to that of nuclear β-catenin, thus providing further evidence of their status as Wnt-related proteins.

SOX9 expression in the atrophic post-menopausal endometrium was up-regulated compared to that of the highly proliferative malignant and proliferative phase endometrium. The proliferation marker Ki67 was also negatively associated with SOX9 expression, suggesting an anti-proliferative role. SOX9 expression appeared to be independent of Wnt activation.

The expression of SOX9 in the Ishikawa endometrial adenocarcinoma cell line can be manipulated by the ovarian sex steroid hormones; treatment with MPA and DHT results in down-regulation. The role of oestrogen and progesterone in regulating the normal cycling endometrium has long been established and has been extensively explored. However, the role of the other ovarian sex steroid hormone, androgen, has been rarely investigated. The cell culture experiments performed here provide preliminary data on a role for androgens in regulating the endometrium.

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UNIVERSITY OF LIVERPOOL

Liverpool Women's NHS
NHS Foundation Trust

Ethics Submission No: 11/H1005/4

PATIENT INFORMATION SHEET

"MIPs in Endometrial Cancer Study"

Role of metastasis-inducing-proteins in endometrial cancer

Version 1.1: For patients undergoing hysterectomy.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

Why are we doing the study?

Each year in the UK about 4500 women, commonly in their 50s and 60s, develop cancer of the lining of the womb (endometrium). The 'endometrium' builds up and is then shed each month as a 'period' before menopause. In women who unfortunately develop cancer, the cells in the endometrium multiply and behave abnormally. The survival rate of early stage endometrial cancer is good, however in extreme cases cancer cells can spread beyond the womb affecting the overall outcome of the disease. We are doing this study to better understand the changes that happen in endometrial cells. This will help us discover new targets to diagnose and design new treatment for endometrial cancer.

What is metastasis?

The spread of cancer cells beyond their origin, the womb, is called 'metastases'. This metastatic process is closely linked with the outcome of the disease, but is not fully understood. It is possible that there are special proteins that can encourage cancer cells to spread. These specific proteins are called 'metastasis-inducing-proteins' (MIPs), which we believe can cause cancer cells to invade healthy tissue. We would like to investigate the presence of these proteins in endometrial cancer cells and the role they play.

Why have I been chosen?

We are looking for a total of 160 women who are undergoing hysterectomy. We are specifically looking for 80 women who have endometrial cancer and another 80 healthy women undergoing surgery. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time and without giving a reason. The decision to not take part or withdraw at any time, will **NOT** affect the standard of care you receive.

What will happen to me if I take part?
Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed from you will be taken for the research.
Therefore, **NO** extra surgery or procedures will be performed for the study. A **blood sample** will also be taken from your veins.

What are the possible benefits of taking part?
We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometrial cancer.

What if something goes wrong?
We do not anticipate any harm to arise while taking part in this study, as we are not carrying out any additional procedures. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action. There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management
The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research, will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it.

Surplus tissue
You can chose to give consent for any remaining tissue, after being used for this study, to be anonymised and stored in the Liverpool Women's Hospital which can be used for future ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact **Mr Jonathan Herod, Consultant Gynaecology Oncologist, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.**

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: The role of metastasis-inducing-proteins (MIP) in endometrial cancer

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree for surplus tissue to be stored in the department of obstetrics & Gynaecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Liverpool Women's
NHS Foundation Trust

Ethics Submission No: 09/H1005/55
PATIENT INFORMATION SHEET

"Endometrial stem cell Study"

The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Version 1: Endometrial biopsy only

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of specials cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells), and their job in the body is not yet determined. The inner-lining of the womb (endometrium) has these stem cells that can become many different types of cells, and they are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 160 women (you must have been off all hormonal medicines for at least 3 months), who have regular periods. We are specifically looking for 80 women who have endometriosis and another 80 completely healthy women who have had at least one baby. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*

   Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.

2. *If you are not having a hysterectomy:*

   Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.

The sample of endometrium will be processed in the lab to isolate the stem cells from it. Therefore, **NO** extra surgery will be performed for the study. A **blood sample** (5mls = teaspoonful of) will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are in the clinic (without anaesthetic) or whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Once we carryout the study on the samples you kindly donate, if there is any surplus tissue, it will be stored in the department of obstetrics & Gyanecology and will be used in other ethically approved studies.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool Women's

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.**

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study and for my GP to be informed of my part taking. ☐
5. I agree for surplus tissue to be stored in the department of obstetrics & Gyanecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

SDS0210M

P.I.S. (February 2005)

Appendix 2: Demographic detail collection form

Endometriosis Studies Clinical Data Collection Form

Sample ID: _____

Date of sample: _____/_____/_____

Sample type: Full thickness/pipelle (circle as appropriate)

Age: _____

Height (m): _____

Weight (kg): _____

BMI: _____

Smoker: yes/no (circle as appropriate)

Endometriosis: yes/no (circle as appropriate)

Endometriosis stage: _____

Adenomyosis: yes/no (circle as appropriate)

Menorrhagia: yes/no (circle as appropriate)

Fibroids: yes/no (circle as appropriate)

Reason for Surgery: _____

Previous gynae history/surgery: _____

Parity: _____ Infertility: _____

Miscarriages: _____ PCOS: yes/no (circle as appropriate)

TOP: _____

Days of bleeding: _____

Cycle Length: _____ Irregular cycle: yes/no (circle as appropriate)

LMP: _____/_____/_____ Menopause: _____/_____/_____

Contraceptive/hormone treatment: _____

Other information: _____

_____

_____

Appendix 3: Positive and negative controls for immunohistochemistry

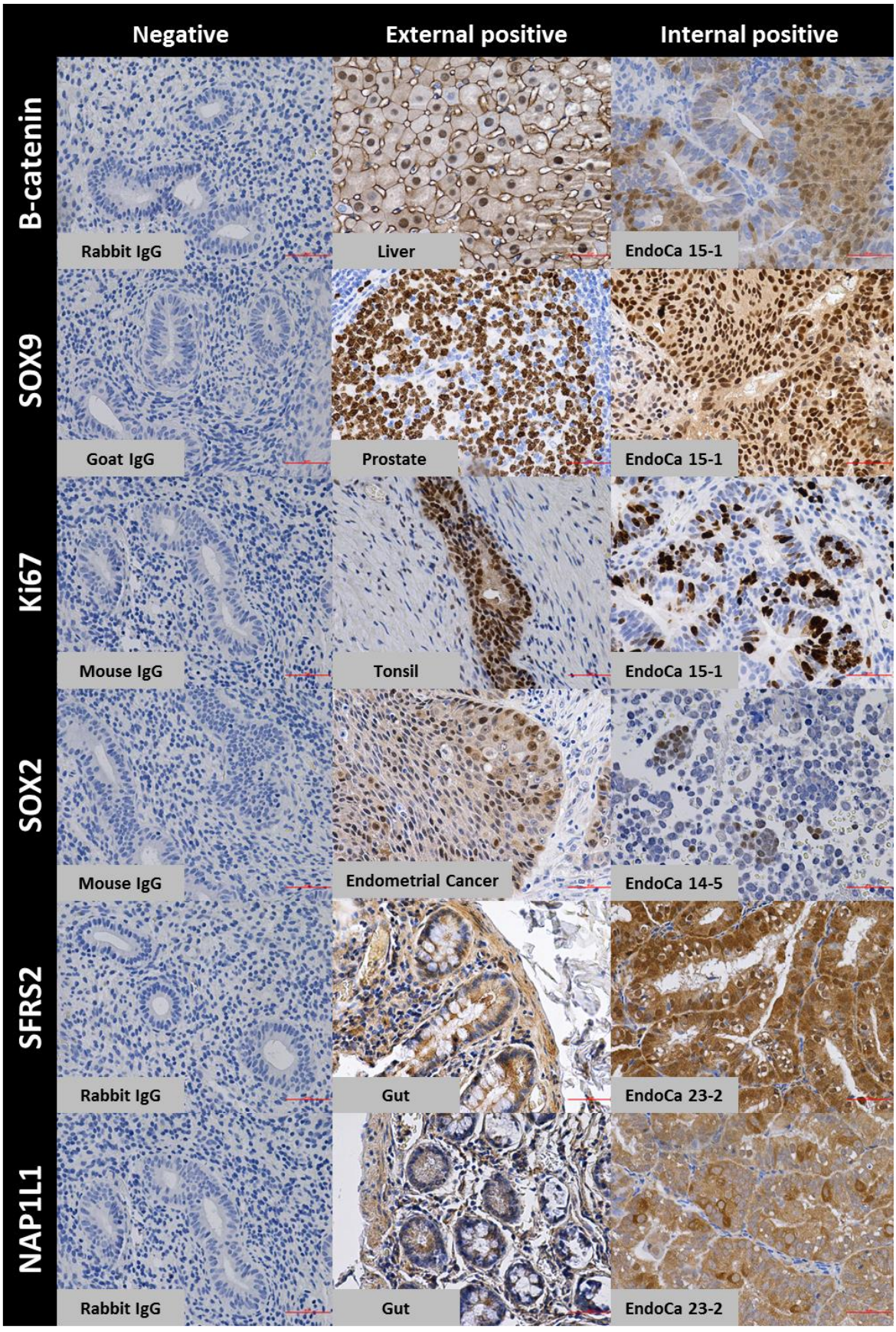

Figure: Controls for immunohistochemistry. Negative control, external positive control and internal positive control. All pictures were taken at 400x magnification.